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Sid.

1. Menzinsky, G., On factors influencing the metabolism and growth of *Saccharomyces cerevisiae* (top yeast) under aerobic conditions 1—94
2. Ställberg-Stenhagen, S., Optically active higher aliphatic compounds. VI. The synthesis of D(—)-21-methyltricosanoic, D(—)-21-methyltetracosanoic, and L(+)-21-methylpentacosanoic acids 95—111
3. Fredga, A. and Bauer, H., Some homologues of tetraethenyl hexasulphide 113—118
4. v. Euler, B. und v. Euler, H., Über spontane Regression implantierter Tumorgewebe 119—124
5. Holmbergh, O., Some observations on the purification of »malt amylase» 125—128
6. Eistert, B., Beiträge zum Redukton-Problem 129—134
7. Hultin, E., Investigations on malt amylase. V. On the stability of α-amylase by lack of calcium 135—141
8. Hultin, E., Investigations on malt amylase. VI. On the purification of α-amylase with tannic acid and acetone 143—159
9. Hultin, E., Investigations on malt amylase. VII. First report on the purification of α-amylase with ammonium sulphate and dialysis 161—171
10. Hultin, E., Investigations on malt amylase. VIII. On the determination of dissociation constants for the compounds of enzymes and their stabilizing metal ions 173—176
11. Jacobson, B., On the adiabatic compressibility of aqueous solutions 177—210
12. Melander, L., On the mechanism of electrophilic aromatic substitution 211—292
13. Lindvall, S. & Carsjö, A., On protein fractions in sea urchin eggs and their changes upon fertilization 293—295
14. v. Euler, H. & Hasselquist, H., Neue Redukton-Derivate 297—303
15. Wideqvist, S., A new method for the calculation of reaction velocity constants 303—311
16. von Euler, H. & Heller, L., Serum fibrinolysin activation in the experimental serum hypersensitiveness of the rabbit 313—319
17. Wideqvist, S., On *tert*-butylsuccinic acid and related compounds 321—324
18. Lindqvist, I., A crystal structure investigation of the paramolybdate ion 325—341
19. Lundblad, G. & Monroy, A., Mucopolysaccharase activity of sea urchin sperms 343—347
20. Lindqvist, I., The structure of the tetramolybdate ion 349—355
21. Cutinelli, C., Ehrensvärd, G. & Reio, L., Acetic acid metabolism in Rhodospirillum rubrum under anaerobic condition 357—361
22. Gustafson, T. & Hjelte, M.-B., The amino-acid metabolism of the developing sea urchin egg 363—366
23. v. Euler, H. & Hasselquist, H., Elektronen-Transport und Stabilisierung bei der Reaktion zwischen Dienen und Philodienen. I 367—372
24. ——, Zur Kenntnis der Reduktone. VI 373—381
25. Wideqvist, S., On the stability of the cyanobenzoic acids in aqueous solution 383—387
26. Porath, J. & Claeson, G., The phase systems fluorobenzoic-chlorobenzoic acids and fluorobenzoic-hydroxybenzoic acids 389—396

Sid.

27. Byström, A. & Wilhelmi, K.-A., The crystal structure of Diaboleite, $Pb_2Cu(OH)_4Cl_2$ . . . . . 397—404

28. Runnström, J. and Monroy, A., The effect of egg extract on the surface of sea urchin eggs and spermatozoa . . . . . 405—416

29. Myrbäck, K., On the amalyse-substrate compounds . . . . . 417—422

30. v. Euler, H., Hasselquist, H. und Glaser, A., Aminoreduktone, Endiamine, Endithiole, Enole und Redukton-Derivate . . . . . 423—429

31. Ställberg-Stenhagen, S., Optically active higher aliphatic compounds. VII. The synthesis of (—)-3(L),6(D)-dimethyltetracosanoic and L(+)6-methylpentacosanoic acids . . . . . 431—442

32. Malm, M. and Wachtmeister, L., A comparison between the potassium content of unfertilized and fertilized sea-urchin eggs . . . . . 443—449

33. Andersson, B., Studies of siccatives. I. Kinetic investigations of the metal-catalysed autoxidation of methyl linoleate . . . . . 451—476

34. Sillén, L. G., On filtration through a sorbent layer. IV. The Ψ condition, a simple approach to the theory of sorption columns . . . . . 477—498

35. Sillén, L. G., On filtration through a sorbent layer. V. »Final form» fronts and broadening fronts. . . . . . 499—512

36. von Euler, H. & Heller, L., Serum fibrinolysin activation in the experimental hypersensitivity of the rabbit. II. Trypsin inhibitor activity . . . . . 513—517

37. Aurivillius, B., Mixed oxides with layer lattices. III. Structure of $BaBi_4Ti_4O_{15}$ 519—527

38. von Euler, B. & von Euler, H., Einflüsse von Wirkstoffen auf Lebensvorgänge normaler und sarkomtragender Ratten . . . . . 529—534

39. Lundgren, G., The crystal structure of $Th(OH)_2SO_4$ . . . . . 535—549

40. Ribi, E., Über den Feinbau der Viscoseseide . . . . . 551—566

41. Holmberg, B., Über Styrylthioglykolsäuren . . . . . 567—579

42. von Euler, H. & Heller, L., Serum fibrinolysin activation in the experimental serum hypersensitiveness of the rabbit. III. Activation experiments with staphylokinase . . . . . 581—585

43. Brändström, A., Use of different alkali carbonates in the methylation of acetylacetone . . . . . 587—597

ARKIV FÖR KEMI Band 2 nr 1

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On factors influencing the metabolism and growth of *Saccharomyces cerevisiae* (top yeast) under aerobic conditions

By Georg Menzinsky

With 7 figures in the text

Table of contents

Preface 2
Introduction 2

Part I. Review. Yield calculations

Chapter I. On the metabolic reactions of growing *Saccharomyces cerevisiae* 3
  1. Review of previous literature 3
  2. Theoretical and experimental yields of yeast in cultivations on sugar 12

Part II. Experiments and Results

Chapter II. Yeast cultivations in the laboratory 19
  1. Cultivations in open fermentation vessels 19
  2. Cultivations in respirometers 24
  3. The effect of substrate concentration 34
  4. The effect of pH 36
  5. The effect of aeration 39
  6. The effect of the seed yeast 39
  7. The effect of amino acids on yeast growth 46
Chapter III. Growth factors and their importance in the growth of yeast 48
  1. Review of previous literature 48
  2. Aerobic laboratory yeast cultivations with addition of various growth factors 51
    a. Addition of biotin 51
    b. Determination of biotin in *Saccharomyces cerevisiae* 53
    c. Biotin contents of yeasts from various cultivation stages and their effects on yeast growth 56
    d. Addition of other growth factors 57
    e. Determination and significance of biotin in molasses 59
    f. The influence of natural extracts on yeast growth 61
Chapter IV. Molasses as a substrate for *Saccharomyces cerevisiae* 63
  1. The chemical composition of molasses 64
    a. Sugars 64
    b. Ash components 64
    c. Organic nitrogen-free non-sugar components 65
    d. Nitrogenous components 66
  2. Analyses of Swedish molasses from different campaigns. The effect on yeast growth of variations in composition of molasses 68
Chapter V 80
  1. Summary 80
  2. Conclusion 84
Appendix. Analytical methods 85
References 90

Preface

The work described here was carried out in the Department of Physiological Chemistry at Wenner-Gren's Institute, University of Stockholm, and in the Central Laboratory of the Swedish Yeast Company (Svenska Jästfabriks A/B) at Rotebro.

To the Director of the Institute, Professor JOHN RUNNSTRÖM, I wish to express my warmest thanks for the interest which he has displayed in my work and for the provision of exemplary laboratory facilities. It is also my pleasant task to thank the Director-General of the Svenska Jästfabriks A/B, Mr. STEN WESTERBERG, for his interest and for his endeavours to maintain the manufacture of compressed yeast in this country at a high level by the support of research programmes. My gratitude is especially due to Mr. HENRY BRAHMER, Technical Manager and Vice-Director of the Svenska Jästfabriks A/B, who has rendered possible, by his constant interest and unflagging support in all respects, the pursuit of this research simultaneously with the performance of my regular duties with the Company. I wish to proffer my thanks to the Swedish Sugar Company (Svenska Sockerfabriks A/B) for their readiness in providing certain research material. In this connection I should especially like to mention my indebtedness to the Head of the Central Laboratory, Mr. O. WIKLUND, and to the Head of the Statistical Bureau of the same Company, Dr. H. ÅSTRAND. For valuable discussions, advice and criticism, my gratitude is due to my valued colleagues Drs. H. BOREI, G. EHRENSVÄRD and E. SPERBER, Mr. H. ANDRÉN and Mr. T. STÅHLE.

Mrs. ASTRI RUNNSTRÖM and Mr. R. STJERNHOLM gave valuable help with the respirometer and Warburg determinations.

The work of translation has been carried out by Dr. D. F. CHEESMAN, for whose interest and patience in this frequently exacting task I wish to express my gratitude.

Introduction

The present investigation is concerned with the metabolic processes in growing yeast of the species *Saccharomyces cerevisiae* (top yeast) under aerobic conditions. It was carried out in order to contribute to the knowledge of the biochemical conditions and factors essential to the growth of compressed yeast.

A study of the literature about the growth of compressed yeast under aerobic conditions showed that the biochemical publications in this field were very scanty. By far the greater part of the papers dealing with yeast are concerned with the carbohydrate metabolism and its relation to the reaction products alcohol and carbon dioxide. Papers actually concerned with the reaction involved in the synthesis of yeast substance are, on the other hand, very few. In addition the majority of these latter investigations were unfortunately carried out under such varied conditions that it is very hard to draw any general conclusions.

The present communication is arranged in the following manner:

The *first part* deals with the chemical processes involved in the synthesis of yeast substance. Generally the literature is considered only in those cases which may have some connection directly or indirectly with the metabolic processes of *Saccharomyces cerevisiae*. Thus only those studies of the *Torula* species, which have some direct connection with the metabolic processes of *Saccharomyces cerevisiae* have been taken into account, especially the works on basal metabolism under aerobic conditions. However, the differences of these two species of yeast are so great —

Torula utilis, for example, requires no added growth factors for its proliferation — that it is impermissible to make too close analogies with regard to the special processes connected with growth and proliferation. The special character of the present work, concerned as it is with the biological synthesis of *Saccharomyces cerevisiae* under aerobic conditions, should, however, justify these omissions.

An interesting subsidiary problem in the study of the yeast synthesis is provided by the theoretical maximum yield of yeast. I am fully aware that this problem is one of extreme difficulty, since our knowledge of the reactions involved in yeast synthesis is very deficient. Nevertheless, an attempt has been made to co-ordinate the various relevant considerations in the literature and to examine them in the light of certain of my own experiences.

The *second part* of the communication is devoted to the experimental investigations. The methods are described by way of introduction. A study is made of the reactions in the biological degradation of sugar in the presence of nitrogenous nutrient materials by means of yeast cultivation experiments in the respirometer. The importance of the substrate concentration, the pH and the aeration in the growth of yeast are discussed in the following sections.

One of the most important aspects of the study of the growth of *Saccharomyces cerevisiae* is the question of the influence of the seed yeast, i.e. the quantities in which it is employed and its physiological state. Matters related to this are treated in some detail in a separate section. The results obtained led to an investigation of the importance of growth factors in the reproduction of yeast. Molasses, as a suitable substrate for the cultivation of *Saccharomyces cerevisiae*, is made the subject of detailed discussion and experimental investigation.

In conclusion, the results of the investigation are summarised and considered with respect to the factors involved determining the growth of *Saccharomyces cerevisiae* under aerobic conditions. No attempt is made at completeness, but the principal aim is to make a contribution to the study of the biological protein synthesis, in the hope that this may provide a stimulus to other workers in the same sphere.

The analytical methods employed are described in an appendix.

PART I

Chapter I. On the metabolic reactions of growing *Saccharomyces cerevisiae*

1. Review of previous literature

Biological syntheses may generally be described as coupled reactions. At the side of, and simultaneously with the syntheses, degradation reactions take place. These usually provide the energy necessary for the syntheses. In the special case of the biological synthesis of yeast substance from carbohydrate, a further characteristic is that the process must be carried on in the presence of atmospheric oxygen, since otherwise the simultaneous degradative reactions will predominate.

In the anaerobic fermentation of sugar by yeast, according to the overall equation

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

100 g glucose will theoretically yield 51.1 g alcohol and 48.9 g carbon dioxide. Now 100 g glucose corresponds to an energy content of 374.3 Cal., 51.1 g alcohol to 364.9

Cal. and the carbon dioxide formed to 0 Cal. The alcohol produced thus has 97.5 % of the energy content of the original sugar. For side-reactions, i.e. syntheses such as occur in yeast growth, only 2.5 % or 9.4 Cal. of the original energy is available.

If, however, the sugar is respired in an aerobic biological system, then the end products of sugar degradation will be water and carbon dioxide, according to the equation

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ H_2O + 6\ CO_2$$

100 g glucose and 106.7 g oxygen thus give 60.0 g water and 146.7 g carbon dioxide. In the energy balance the reaction products water and carbon dioxide correspond to 0 Cal. Hence, under these conditions, the total energy of the original sugar, 374.3 Cal. per 100 g glucose, is utilisable energy. This energy is available for other parallel and simultaneous synthetic reactions (cf. FINK (1938 c)).

These two reactions prevail in the synthesis of biological materials by various types of aerobic yeast, for example in the growth of *Saccharomyces cerevisiae* (top yeast) where the yeast synthesis proceeds from carbohydrates and nitrogenous nutrient salts during strong aeration of the substrate. For the study of the metabolic processes in the growth of yeast, it is necessary to know the chemical composition of the organism. There are yeasts with about 12 % protein, ENEBO, ANDERSON and LUNDIN (1946), while others have up to 75 % protein, NIELSEN (1933). The commercial yeast put on the market by the compressed yeast industry usually has an average protein content of 40–50 %.

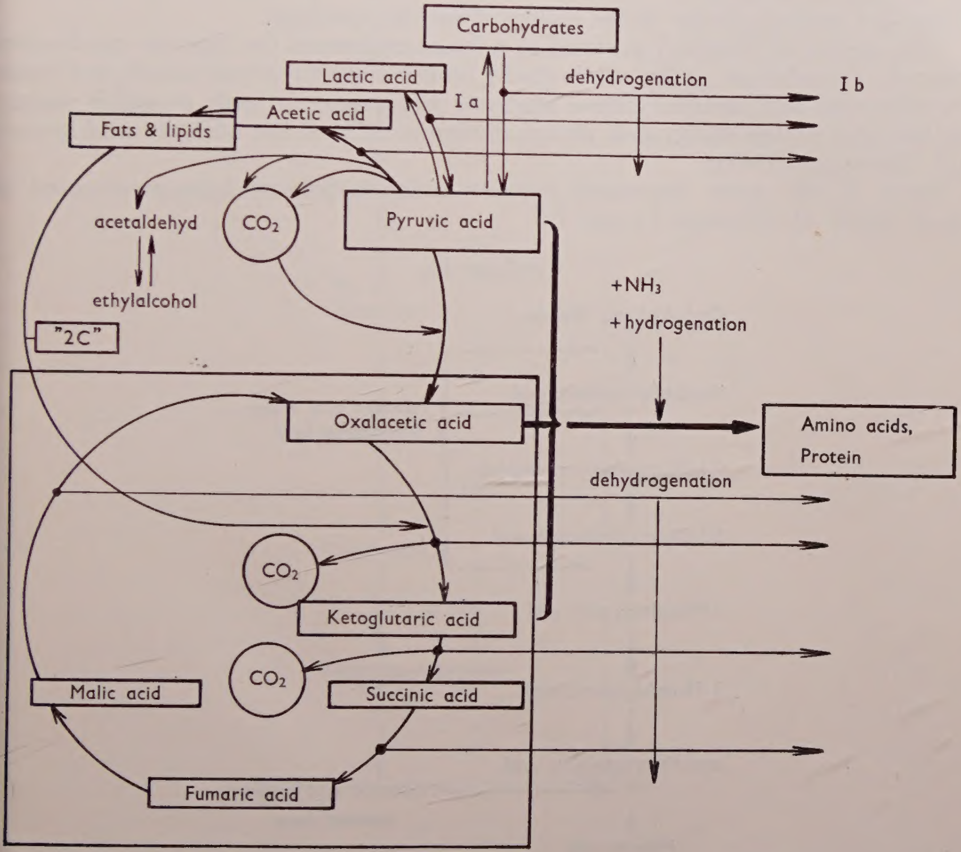
Little is yet known with certainty of the chemical processes involved in the synthesis of yeast substance from sugar and nutrient salts. It is common to speak of yeast protein, which tends to give the impression that a single well-defined substance is involved. As, however, this is by no means the case, it would be preferable to employ the term "yeast proteins".

Even though the structure of the protein molecule is unknown, it is well established that every protein, on degradation, gives a number of more or less well-defined split products. Among these the amino acids occupy an especially prominent position. The pathways taken by amino acids in their synthesis to proteins are very incompletely known, but experiments with isotopes of carbon have shown that the carbon skeletons of the original amino acids can be detected in the proteins formed by biological synthesis. It may therefore be presumed, albeit with some reservation, that the amino acids constitute the raw material of proteins and hence the first stage in the protein synthesis in yeast. It is probable that, in condensation of the amino acids to protein, glutamine and asparagine, as well as the energy-yielding phosphate cycles, play an important part.

Survey of the processes of carbohydrate metabolism

In order to understand the formation of the raw materials of protein synthesis, the amino acids, it is necessary to consider the process of carbohydrate degradation, since in this the nitrogen-free basic skeletons of the amino acids are formed, the latter being ultimately produced by amination processes. In studies of the aerobic degradation of pyruvic acid, it has been shown that this is a cyclic process during which the pyruvic acid undergoes a step-wise decarboxylation. Each carbon atom is split off as carbon dioxide, while the hydrogen, by dehydrogenation processes and subsequent oxidation over the cytochrome system, is converted to water. In cases where the different stages of this cyclic process are known, the course of degradation appears to proceed in a manner represented in a simplified form in Scheme I.

Scheme I


Carbohydrates
Lactic acid
Acetic acid
Fats & lipids
I a
dehydrogenation
I b
acetaldehyd
ethylalcohol
CO_2
Pyruvic acid
"2C"
+NH₃
+hydrogenation
Oxalacetic acid
Amino acids, Protein
dehydrogenation
CO_2
Ketoglutaric acid
CO_2
Malic acid
Succinic acid
Fumaric acid

It should be emphasised at this point that the experimental work upon which the scheme is based has partly been carried out with animal material, so that the results are not immediately applicable to yeast. Nevertheless, as far as *the carbohydrate degradation* is concerned, biochemical work has shown great similarities between the enzymic processes in animal cells and in yeast cells. It may be mentioned that, making use of the extreme adaptability of *Torula* yeast, Ehrensvärd and coworkers (J. Biol. Chem. 1949, in press, and Acta Chem. Scand. (1949)), have been able to show that acetic acid, as sole carbon substrate for the growth of *Torula* yeast, seems to be utilized aerobically according to Scheme I. In general this work seems to confirm for *Torula* yeast the findings of Lifson, Lorber, Sakami and Wood (1948) for the acetic acid metabolism in rat liver. It would therefore appear justified to consider the main features of the scheme as being applicable to yeast in general.

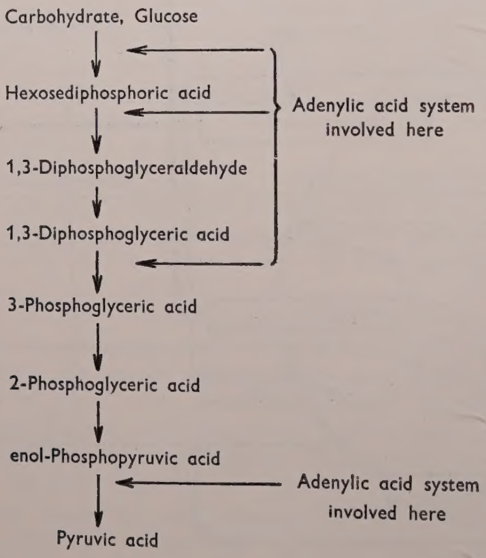
(As long ago as in 1926, OPPENHEIMER considered the connection between aerobiosis and anaerobiosis in animals and the higher plants to be fundamentally similar to that in yeasts.)

The same conception of metabolic processes in animal and vegetable cells will also be adopted in the following sections with respect to the synthesis and degradation of the *protein components*. It would seem that this is justified for the purpose of forming a general picture of the biological protein synthesis.

The portion of Scheme I enclosed in a frame represents the separate mechanism whereby the substrate of the cells is finally broken down to carbon dioxide and water by reduction and decarboxylation processes and energy is made available mainly in the form of the energy-rich phosphate compounds of the adenylic acid system (cf. LIPPMANN (1941)).

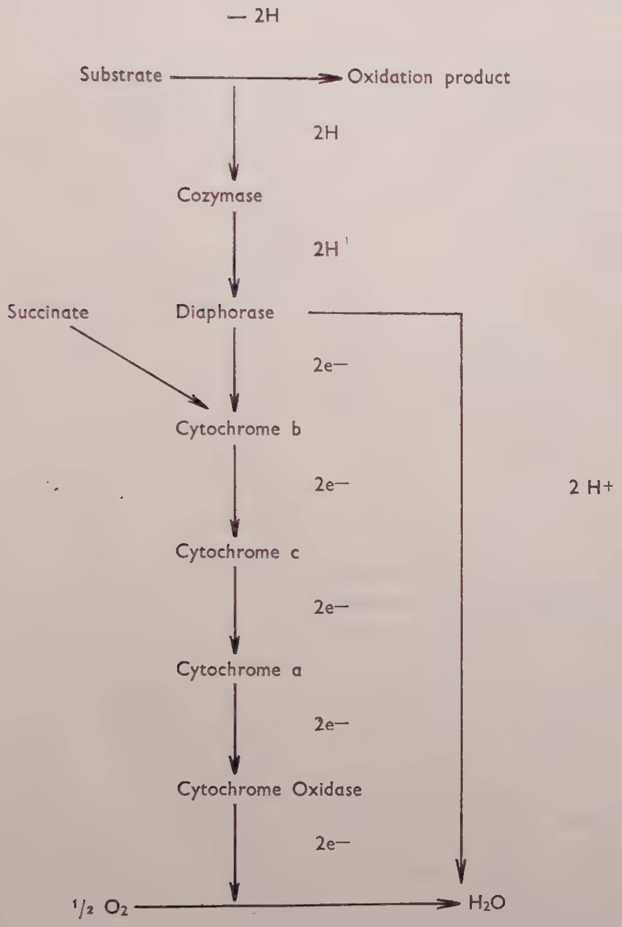
Some of the more important reaction series in Scheme I are represented in more detail by Schemes I a and I b.

Scheme I a


Carbohydrate, Glucose
Hexosediphosphoric acid
1,3-Diphosphoglyceraldehyde
1,3-Diphosphoglyceric acid
3-Phosphoglyceric acid
2-Phosphoglyceric acid
enol-Phosphopyruvic acid
Pyruvic acid
Adenylic acid system involved here
Adenylic acid system involved here

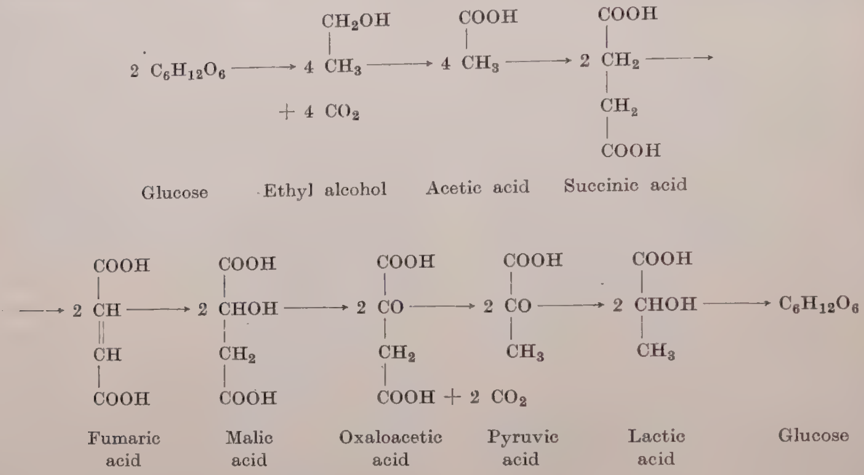
The substrate is oxidised (I b), by which process energy is made available to the cell. The step-wise nature of the oxidation process over the consecutively acting enzymes ("carriers") permits an efficient regulation of the liberation of energy. The cytochrome system must be regarded as the main path of all final oxidative processes in the yeast cell. This system can utilise as substrates the most varied intermediate products of the carbohydrate degradation and is thus capable of delivering energy at many points in the metabolic cycles of the living cell. It should be recalled that proteins and fats also have degradation products, in certain cases the same products as those from the carbohydrate degradation, which may undergo oxidative degradation in the same manner as the latter for the provision of energy. A detailed discussion of the cytochrome system is to be found in the monograph of BOREI (1945), among other authors.

Scheme I b


— 2H
Substrate
Oxidation product
2H
Cozymase
2H
Succinate
Diaphorase
2e—
Cytochrome b
2e—
2 H+
Cytochrome c
2e—
Cytochrome a
2e—
Cytochrome Oxidase
2e—
½ O₂
H₂O

The question of the aerobic degradation of carbohydrates by yeast cells is dealt with in earlier papers by Effront (1927), Claassen (1934 a) and Bernhauer (1933). The latter author maintains that aerobic fermentation processes must invariably be preceded by an anaerobic breakdown of sugar molecule, since the aerobic oxidative processes can only enter by way of certain intermediate products. According to this theory, the anaerobic sugar degradation gives rise partly to the formation of lactic acid and partly, over succinic acid, to acetic acid and alcohol. In certain animal cell processes the formation of lactic acid is of great importance, e.g. in muscular work. According to Hohl and Joslyn (1941) there is also a small formation of lactic acid in the yeast cell. The work of Lieben (1923), Lundin (1923), and Myrbäck and Everitt (1924) has shown that in yeast, under strongly aerobic conditions, a resynthesis occurs from the end products of the sugar degradation: thus alcohol, lactic acid and acetic acid are resynthesised to carbohydrates. In this process, however, the intermediate products have not been identified, but only the final state as reflected in the increase in the yeast substance.

BERNHAUER (1933) has suggested the following scheme for this resynthesis of carbohydrate:


2 C6H12O6
4 CH2OH–CH3 + 4 CO2
4 COOH–CH3
2 COOH–CH2–CH2–COOH
Glucose
Ethyl alcohol
Acetic acid
Succinic acid
2 COOH–CH=CH–COOH
2 COOH–CHOH–CH2–COOH
2 COOH–CO–CH2–COOH + 2 CO2
2 COOH–CO–CH3
2 COOH–CHOH–CH3
C6H12O6
Fumaric acid
Malic acid
Oxaloacetic acid
Pyruvic acid
Lactic acid
Glucose

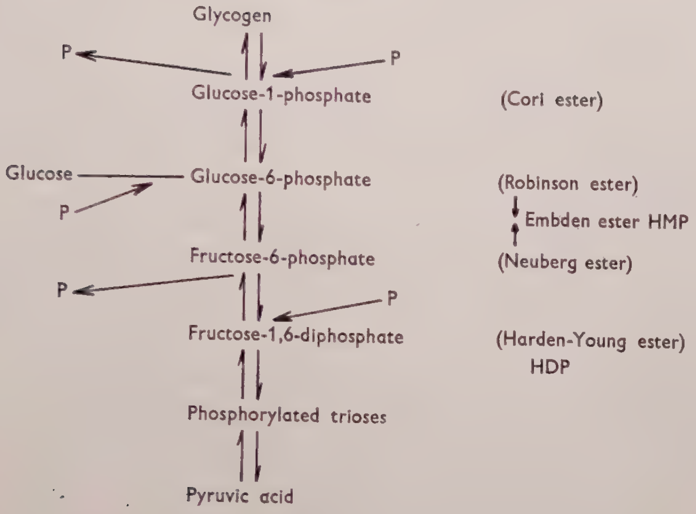
It is to be noted that this scheme has many points in common with the four-carbon cycle of SZENT-GYÖRGYI (1925). Its connection is also evident with the aerobic sugar degradation.

Among the nitrogen-free substances in yeast, the polymeric reserve carbohydrates occupy a position of special interest. In his discussion of this matter, CLAASSEN (1934 a) treats these as being of the glycogen type and considers them to constitute not structural materials but reserve substances, stored by the yeast when the nutrient solution is rich in sugar and only slightly aerated. CLAASSEN conceives this storage process as a direct polymerisation of glucose, a depolymerisation taking place when the nutrient medium is deficient in sugar. The uptake of glucose with the formation of reserve carbohydrate is thus not a true assimilation process, in which a stable structural material is produced, but rather a transient storage of sugar that cannot immediately be dealt with by the yeast on account of a temporary excess of this substance. Mention must be made in this connection of the studies by MYRBÄCK (1936, 1937), MYRBÄCK and ÖRTENBLAD (1936, 1937 a, 1937 b), and BRANDT (1941) on the role of trehalose in the storage of carbohydrate by yeast. BRANDT showed that in growing yeast, which was provided with assimilable nitrogen in addition to glucose, the contents of trehalose and glycogen were significantly lower than in non-growing, mature yeast. It was tempting to suppose that trehalose was associated with the metabolism characteristic of growing yeast.

The mechanism of the participation of trehalose in carbohydrate storage in yeast has not, however, been completely investigated, so that it will not be treated in detail at the present juncture.

Scheme I c shows the main features of the synthesis and degradation of glycogen, as compiled from the monographs of POTTER (1944) and STOTZ (1945).

Scheme I c


Glycogen
P
P
Glucose-1-phosphate
(Cori ester)
Glucose
P
Glucose-6-phosphate
(Robinson ester)
Embden ester HMP
Fructose-6-phosphate
(Neuberg ester)
P
P
Fructose-1,6-diphosphate
(Harden-Young ester)
HDP
Phosphorylated trioses
Pyruvic acid

Review of the processes of protein metabolism

Until quite recently very little was known of the connection of the aerobic carbohydrate degradation with protein synthesis. Braunstein and Kritzman (1937 a, b) and Braunstein (1947) have shown, in their studies of the transamination process, the connection of the compounds of the carbohydrate metabolism with the nitrogenous compounds partaking in the protein metabolism, namely the amino acids. They suggest that tissues are able to synthesise amino acids from the corresponding keto acids if one of the dicarboxylic amino acids donates the amino group. Later Cohen (1942) and Lichstein and Cohen (1945) have shown that many bacteria can carry out such transamination reactions.

The fundamental work on the chemistry of proteins, their synthesis and degradation, dates back to Emil Fischer. He showed for the first time that proteins are built up of α-amino acids, coupled together by peptide bonds between the amino and carboxyl groups. The peptide bonds are split by hydrolysis with the formation of free amino acids. It is generally assumed that the synthesis of proteins corresponds to the hydrolytic degradation in the reversed sense.

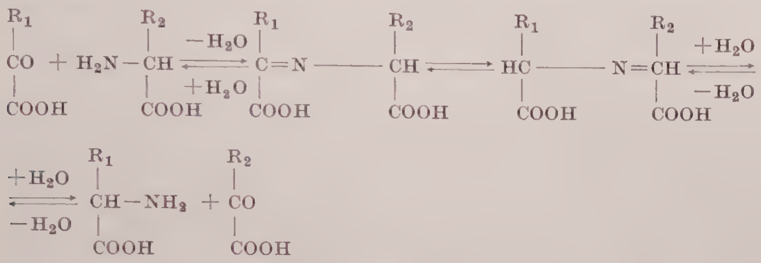
Neubauer and Fromherz (1911) and Flatow (1910) succeeded in demonstrating, in the animal organism, the conversion of amino acids to the corresponding keto acids. Thus the first step in the degradation of an amino acid is an oxidative deamination with the formation of a keto acid. This reaction was believed by Krebs (1932, 1933 a, b, 1935 a, b, c) to be enzymic in character and he ascribed the effect to amino acid deaminases. According to this author there are two different amino acid oxidodeaminases, first a d-amino acid deaminase, which only degrades the unnatural optical isomers of the amino acids, and secondly a l-amino acid deaminase, which attacks the natural amino acids. Edlbacher and Grauer (1944 a, b) detected several l-amino acid oxidases in the animal organism and showed that l-alanine, l-asparagine, l-phenylalanine and l-glutamic acid were degraded by different enzymes. BLANCHARD, GREEN and co-workers (1944, 1945) isolated an enzyme from animal tissues which performed the oxidative degradation of a large number of natural amino acids, leucine, methionine, proline, norleucine, norvaline, phenylalanine, tryptophane, isoleucine, tyrosine, valine, histidine, cystine and alanine. Except in the case of proline, which underwent a ring fission with the formation of α-amino-δ-ketovaleric acid, the corresponding keto acids were formed. Further investigations, which led to the isolation of l-amino acid oxidases from animal tissues, acting by deamination and the formation of keto acids, were carried out by STUMPF and GREEN (1944) and ZELLER and MARITZ (1944, 1945 a, b).

l-Glutamic acid occupies a special position among the l-amino acids, since it is degraded by different biological materials which, however, have no action on other amino acids. EULER *et al.* (1937, 1938), ADLER *et al.* (1938 a, b) and DEWAN (1938) found in various animal and vegetable materials, as well as in yeast and bacteria, an enzyme, glutamic acid dehydrogenase, which catalysed the conversion of l-glutamic acid to ketoglutaric acid and ammonia. It proved to be strictly specific for glutamic acid as substrate. It was also remarkable that the reaction was reversible, a phenomenon apparently unique among the amino acid oxidases.

The synthesis of glutamic acid, however, requires not only the presence of ketoglutaric acid and ammonia, but also a hydrogen donator in the form of a dihydrocozymase. A similar system is to be found in the KREBS cycle (1937, 1943), where it is assumed to be responsible for a part of the main course of carbohydrate degradation (cf. the framed portion of Scheme I, p. 5). QUASTEL and WOOLF (1926) report an enzyme occurring in bacteria, aspartase, which is able to synthesise aspartic acid from fumaric acid and ammonia. VIRTANEN and TARNANEN (1932) succeeded in demonstrating this synthesis experimentally in a cell-free extract of bacteria. The same enzyme was subsequently detected in yeast by HAEHN and LEOPOLD (1937). DAS (1945), in connection with his work on the biological synthesis of amino acids by *B. coli aerogenes*, emphasises the importance of oxaloacetic acid in this process. He believes that aspartic and glutamic acids are the primary products, these giving rise to other amino acids by transamination.

Of especial interest for the understanding of the biological protein synthesis are the investigations by BRAUNSTEIN and KRITZMANN (1937 a, b) of transamination reactions giving rise to the formation of amino acids. These authors found that in animal tissues glutamic and aspartic acids react with keto acids in such a manner that ketoglutaric acid or oxaloacetic acid respectively is produced, together with the amino acid corresponding to the original keto acids. These reactions are dealt with in great detail by, among others, BRAUNSTEIN (1939, 1947), COHEN (1942) and HERBST (1944). It is therefore unnecessary to go further into the matter here. The different workers are still not agreed as to the extent to which the phenomenon actually occurs. Originally BRAUNSTEIN was of the opinion that transamination could occur between any l-amino acid and ketoglutaric acid. Later, BRAUNSTEIN and ASARKH (1945) stated that only aspartic acid, glutamic acid, alanine, cysteine, valine and leucine were capable of entering into transamination reactions. COHEN (1942) limited the reaction still further, maintaining that only glutamic acid, aspartic acid and alanine could enter into transaminations. ROINE (1947) has succeeded in demonstrating, in baker's yeast, the transamination reaction glutamic acid + + oxaloacetic acid $\rightleftarrows$ ketoglutaric acid + aspartic acid, and in *Torula utilis* the transamination of valine, leucine and isoleucine with ketoglutaric acid.

The reaction mechanism proposed by BRAUNSTEIN and KRITZMANN for the enzymic transamination is analogous to the scheme of Herbst for "non-biological" transaminations (1934, 1944).



The intramolecular displacement of the carbon-nitrogen double bond may correspond to the enzymic step.

Comprehensive work on the enzymic transamination reaction has been carried out in recent years by Cohen (1942), Green, Leloir and Nocito (1945), Schlenk and Snell (1945), Lichstein and Cohen (1945), Lichstein, Gunsalus and Umbreit (1945), Braunstein, Kritzmann, Gale and Tomlinson (1946), among other authors. Braunstein believes in the existence of two different transaminating enzymes, glutamic acid aminopherase (more usually called transaminase), which catalyses the reactions glutamic acid ⇄ alanine and glutamic acid ⇄ aspartic acid, and aspartic acid aminopherase, catalysing the reaction aspartic acid ⇄ alanine. Green *et al.* (1945) are of the opinion that the reactions catalysed, according to Braunstein, by the glutamic acid aminopherase, are, in fact, brought about by two different enzymes. They have isolated these two transaminating enzymes from animal material and named them glutamic acid aspartic acid transaminase and glutamic acid alanine transaminase. These would seem to correspond together to the Braunstein glutamic acid aminopherase.

Even if the transamination processes described above for the formation of alanine, glutamic acid and aspartic acid presumably constitute the main path for the formation of the primary nitrogenous materials from the degradation products of the carbohydrates, there certainly exist other transamination processes in biological systems, such as the transamination demonstrated by Roine (loc. cit.) in *Torula utilis* between valine, leucine and isoleucine on the one hand and ketoglutaric acid on the other. All these transferences of nitrogen from amino acids to keto acids give the impression that the nitrogen in living organisms is freely distributed among all the nitrogen-containing compounds. In a series of investigations, Schoenheimer and co-workers (cf. Schoenheimer 1940, 1946) showed that any amino acid in which the amino nitrogen was labelled with heavy nitrogen, transferred its nitrogen to other amino acids when incorporated in living cells, with a few exceptions, e.g. lysine.

The carbon skeletons of aspartic acid, alanine and glutamic acid, derive, as has already been mentioned, from the corresponding keto acids. The sources of the carbon skeletons of the other amino acids are known with less certainty. Investigations with the heavy isotopes of carbon and nitrogen have shown several other transitions, e.g. from serine to glycine and conversely. It is known from work with *Neurospora* that serine is one of the starting materials in the formation of tryptophane, Tatum and Bonner (1944).

In the treatment of these highly complicated questions, the part played by the constantly formed carbon dioxide should not be forgotten. Werkman and Wood

(1942) have shown that a part of the carbon dioxide split off in the carbohydrate degradation by bacteria (propionic acid bacteria) is again bound in the form of carboxyl groups, subsequently appearing as, among other things, keto acids, which are in equilibrium with the corresponding amino acids. EHRENSVÄRD and co-workers (1948) have shown, for yeast, that *Torula utilis*, cultivated in an atmosphere of heavy carbon dioxide, takes up considerable quantities of this material in the carboxyl groups of the amino acids. Hence carbon dioxide appears to be as important as nitrogen for the formation of the different amino acids. It follows that the amino acid turnover is a transitional stage in the protein synthesis. In other words, the amino acids are not only to be considered as end products, or as material for the synthesis of proteins, but also as substances which, after deamination and oxidative degradation, provide the nitrogen-free material for the energy-yielding dehydrogenation processes. Little is known with certainty regarding the actual synthesis of proteins from the amino acids. Two recent views of the mechanism of the synthesis may nevertheless be mentioned. LIPPMANN (1941) has considered the possibility that the amino acids are phosphorylated at the carboxyl group before condensation occurs. LINDERSTRØM-LANG (1939) and HERBST and SHEMIN (1943) point out the possibility that substances other than the amino acids, e.g. glyoxals, may partake in the formation of peptide chains.

By way of conclusion to the above considerations, Scheme II presents a general survey of the relations between protein synthesis and the carbohydrate and amino acid metabolisms.

In the consideration of the above scheme, it must be borne in mind that the greater part of the reactions involved are reversible. This implies, among other things, that the protein may likewise be degraded to amino acids by enzymic reactions. This is, indeed, already expressed in the term "dynamic equilibrium of proteins", SCHOENHEIMER (1946).

2. Theoretical and experimental yields of yeast in cultivations on sugar

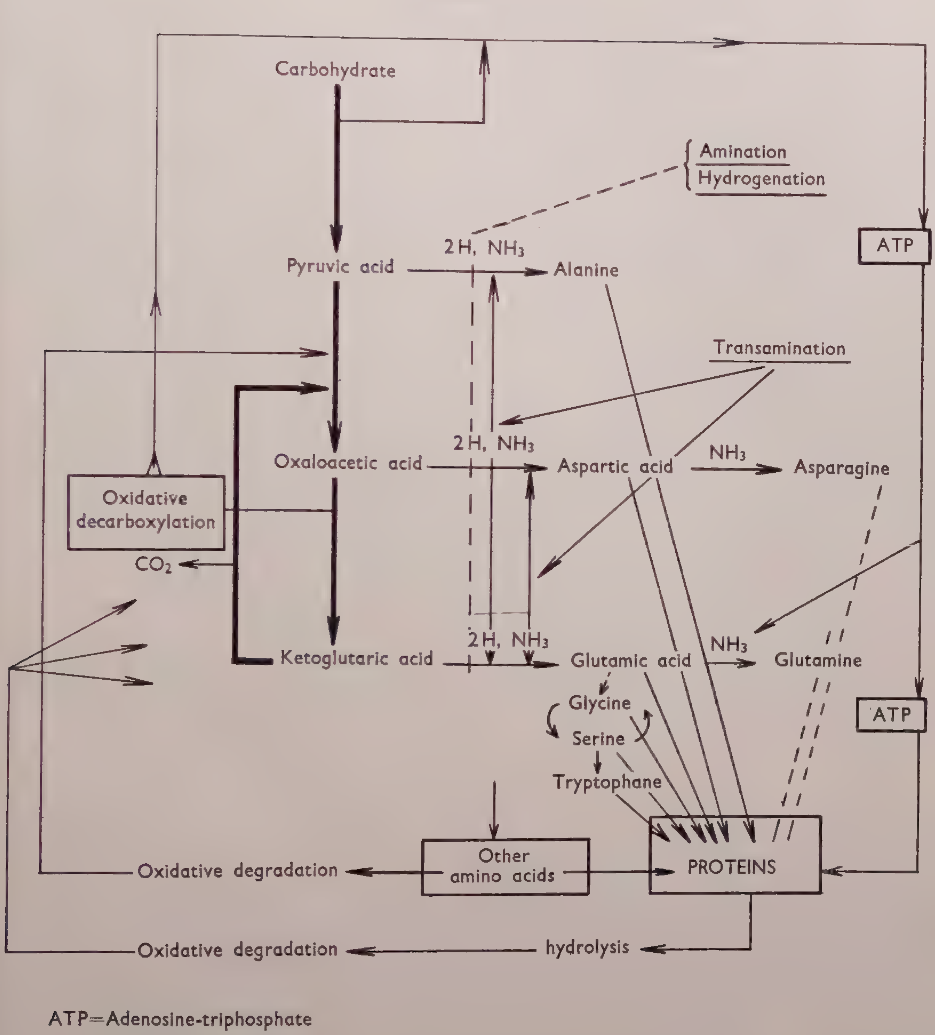
The problem of the theoretical, or in other words maximum, yield in the biological synthesis of yeast has not yet received a completely satisfactory solution. The reason for this lies in the difficulties always met with in the consideration of living experimental materials. In living organisms the biochemical processes are determined by innumerable enzyme-catalysed reactions, which renders it impossible to express the growth process by an unambiguous series of chemical equations.

In the following account, only those yeast yields will be considered which can be attained in laboratory cultivation experiments with sugar as the only carbon source and inorganic ammonium salts as the only sources of nitrogen.

In the treatment of the question of yield, it is first essential to be clear as to the definition of the term. In the present work, a "stoichiometric yield" will be taken to mean that calculated on the basis of stoichiometric relationships. The expression "maximum biological yield" will be applied to that yield which can be attained after consideration of all the chemical and biological reactions in the yeast synthesis and under favourable experimental conditions. By "actual maximum yield" will be understood that already obtained under experimental conditions previously recognised as favourable.

During the last decades several experiments have been made in order to gain some idea, on the basis of chemical equations, of the theoretical yield of yeast substance in the biological yeast synthesis.

Scheme II


Carbohydrate
Amination
Hydrogenation
ATP
2 H, NH₃
Pyruvic acid
Alanine
Transamination
2 H, NH₃
Oxaloacetic acid
Aspartic acid
NH₃
Asparagine
Oxidative decarboxylation
CO₂
2 H, NH₃
Ketoglutaric acid
Glutamic acid
NH₃
Glutamine
Glycine
Serine
Tryptophane
ATP
Other amino acids
PROTEINS
Oxidative degradation
Oxidative degradation
hydrolysis
ATP=Adenosine-triphosphate

One of the best known experiments was based on the equation of EFFRONT (1927) for the synthesis of yeast protein from sugar and nitrogen:

$$3\ C_6H_{12}O_6 + 3\ O_2 = 6\ CH_3CHO + 6\ CO_2 + 6\ H_2O$$
$$6\ CH_3CHO + 3\ N = C_{12}H_{20}N_3O_4 + 2\ H_2O$$
$$\overline{3\ C_6H_{12}O_6 + 3\ O_2 + 3\ N = C_{12}H_{20}N_3O_4 + 6\ CO_2 + 8\ H_2O}$$

Glucose	Yeast protein
540	270
2	1

According to this equation two parts of glucose are necessary for the synthesis of one part of yeast protein. Yeast consists, however, not only of protein, but also of polysaccharides. These, according to EFFRONT, are synthesised directly by the cells by polymerisation of sugar, without any preceding breakdown. One part of sugar would thus be necessary for the synthesis of one part of yeast carbohydrate. Under the assumption that yeast consists of 50 % protein and 50 % carbohydrate, 66.7 g of ash-free dry yeast substance should therefore be obtained from 100 g glucose. With an average ash content of 8 % in yeast, this corresponds to 72.4 g dry yeast substance per 100 g glucose. In his experiments EFFRONT obtained from 100 g glucose, 56 g dry yeast substance with 49.1 % protein and 41.8 % nitrogen-free material. EFFRONT explains the excessive consumption of glucose by supposing that in the formation of yeast substance a certain amount of glucose is burnt for the generation of energy. The equations of EFFRONT must, by his own admission, be regarded as purely schematic in character.

CLAASSEN (1934 a), in his treatment of EFFRONT's hypothesis first derived formulae for the calculation of the theoretical yeast yield from certain theoretical considerations of the aerobic and anaerobic degradation of carbohydrate, supported by experimental findings. Where the yeast protein synthesis was concerned, CLAASSEN found himself in complete agreement with EFFRONT. Regarding the synthesis of carbohydrate in the yeast cell, however, his opinion differed from that of the other author. In this connection he points out the difference in the compositions of glucose ($C_6H_{12}O_6$) and of polysaccharide $(C_6H_{10}O_5)_n$ and supposes, as did BERNHAUER (1933) that two molecules of $C_6H_{12}O_6$ are required in the living cell for the synthesis of one unit of polysaccharide corresponding to the formula $C_6H_{10}O_5$. By this assumption, one part of polysaccharide would be formed in the yeast cell from 2.22 parts of glucose. CLAASSEN gives the following formula for the calculation of the stoichiometric yield of yeast from 100 g sugar:

$$\text{Yeast crop } \% = \frac{100}{2 \times \text{protein} + 2.22 \times \text{N-free}} \times 100.$$

Here "protein" indicates the protein content of the yeast in % and "N-free" the content of nitrogen-free material of the yeast in %. For a yeast composition of 55 % protein, 37 % nitrogenfree substances and 8 % ash, CLAASSEN calculates the yeast yield from 100 kg glucose to be 52.1 kg dry weight. He has not, however, taken the ash content into consideration. If alcohol is also formed in the yeast synthesis, the sugar required for this, corresponding to double the weight of the alcohol, must be subtracted. CLAASSEN also corrects for the non-assimilable nitrogenous metabolites of the yeast, which he estimates to 7 % of protein formed. He then gives for the calculation of the maximum biological yield of yeast from 100 g sugar the formula:

$$\text{Yeast crop } \% = \frac{100 - 2 \times \text{alcohol}}{2.14 \times \text{protein} + 2.22 \times \text{N-free}} \times 100.$$

Here "alcohol" indicates the amount of alcohol formed by 100 g sugar, "protein" the protein content of the yeast in % and "N-free" the content of nitrogen-free material of the yeast in %. According to this formula he finds that 45.0 kg dry yeast substance will be formed per 100 kg sugar, when the composition of the yeast is 55 % protein and 37 % N-free material, and when 10 % of the sugar is fermented to alcohol.

In a series of publications on the biological synthesis of cell substance FINK and co-workers (1938 a, 1939) have expressed their opinion of CLAASSEN's formula and also discussed the stoichiometric and maximum biological yields in the biological protein synthesis. In contradistinction to CLAASSEN, who works with *Saccharomyces cerevisiae*, FINK *et al.* employ *Torula utilis*, for which reason the results of the two schools are not immediately comparable. Nevertheless the work of FINK is an interesting contribution to the problem of the stoichiometric, maximum biological and actual maximum yields of yeast. For this reason it should be discussed here. In 1938 FINK established experimentally that a maximum of 53.7 kg of dry yeast substance (*Torula utilis*) can be obtained per 100 kg of glucose consumed. CLAASSEN (1934 b) gives the figure 54.7 kg of dry yeast substance per 100 kg of sugar. This figure, however, has no experimental support, but has been calculated from general theoretical considerations and practical experience. In 1939, FINK and KREBS criticised CLAASSEN's view, supported by the theory of BERNHAUER, that two parts of sugar were necessary for the storage of one part of nitrogen-free material in the yeast cell. FINK imagined the synthesis of the nitrogen-free components of yeast to proceed according to the overall equations:

$$\begin{array}{ll} \text{I} & 3\ C_6H_{12}O_6 + 3\ O_2 \rightarrow 6\ CH_3CHO + 6\ CO_2 + 6\ H_2O \\ \text{II} & 6\ CH_3CHO + 3\ O_2 \rightarrow 2\ C_6H_{12}O_6 \\ \text{III} & 2\ C_6H_{12}O_6 \qquad\qquad \rightarrow 2\ (C_6H_{10}O_5) + 2\ H_2O \end{array}$$

According to these equations 1.67 parts of sugar would be necessary for the production of one part of nitrogen-free material. FINK then gave the following formula for the calculation of the stoichiometric yield:

$$\text{Percentage yield of yeast} = \frac{100 \times \text{increase in dry yeast substance}}{2.00 \times \text{increase in prot.} + 1.67 \times \text{g ash and N-free}}$$

With this formula FINK obtains the figure 58.5 g as the stoichiometric yield of dry yeast substance from 100 g of glucose. For the calculation of the maximum biological yield from the increases in protein and nitrogen-free material with allowance for the alcohol formed and the reliberated protein, FINK gives the formula:

$$\text{Percentage yield of yeast} =$$

$$= \frac{100 \times \text{increase in dry yeast substance}}{(\text{g prot.} + \text{g exc. prot.}) \times 2.00 + (\text{g ash} + \text{g N-free}) \times 1.67 + \text{g alcohol} \times 1.96}$$

With the aid of this formula FINK arrives at the value 54.4 kg for the maximum biological yield of yeast from 100 kg of sugar. In this connection reference may be made to the work of SPERBER (1945) who showed, by a number of yeast cultivation experiments with *Torula utilis* in the respirometer, that 2.12 g glucose was necessary for the formation of 1 g of nitrogen-free organic yeast substance and 2.10 g glucose for the production of 1 g yeast protein.

Another mode of calculating both the stoichiometric and the maximum biological yields of yeast, differing from that of the authors mentioned above, has been reported by Brahmer (1932, 1947). On the basis of several elementary analyses of different types of yeast, such as compressed yeasts cultivated under more or less strongly aerobic conditions and on different substrates such as grain mashes, molasses and wood sugar worts, this author was able to demonstrate that variations in the composition of the yeast were only appreciable with respect to the nitrogen and ash contents. The percentage compositions with regard to carbon, hydrogen and oxygen were relatively constant. As average values for the different types of yeast, Brahmer reports the following values: $C = 46.28\,\%$, $H = 6.49\,\%$, $O = 30.94\,\%$, $N = 8.43\,\%$ and ash $= 7.86\,\%$. If these values are recalculated for ash-free material the values obtained are: $C = 50.23\,\%$, $H = 7.04\,\%$, $O = 33.58\,\%$ and $N = 9.15\,\%$. If the nitrogen is now eliminated, we obtain: $C = 55.29\,\%$, $H = 7.75\,\%$ and $O = 36.97\,\%$. There thus remains a nitrogen-free and ash-free basic substance, consisting only of C, H and O, which shows a very constant composition for all the types of yeast investigated. This leads Brahmer to imagine a CHO yeast with the composition 55.29 % C, 7.75 % H and 36.97 % O, corresponding closely to the formula $C_6H_{10}O_3$ ($C = 55.39\,\%$, $H = 7.69\,\%$, $O = 36.92\,\%$).

This CHO yeast may be derived from glucose as follows:

$$\begin{array}{ll} 1. & 6\ C_6H_{12}O_6 = 6\ C_6H_{10}O_3 + 6\ H_2O + 6\ O_2 \\ 2. & \quad C_6H_{12}O_6 + 6\ O_2 = 6\ CO_2 + 6\ H_2O \\ \hline & 7\ C_6H_{12}O_6 = 6\ C_6H_{10}O_3 + 6\ CO_2 + 12\ H_2O \end{array}$$

According to this, 61.9 parts of CHO yeast are obtained from 100 parts of glucose. If the CHO yeast is complemented with quantities of nitrogen and ash corresponding to the average values found in the elementary analyses, namely 7.86 % ash and 8.43 % nitrogen, we have 74.0 g of complete yeast, which Brahmer considers to be the stoichiometric yield of dry yeast substance from 100 g glucose.

In practice, however, such a yield is unattainable, since at the side of, and simultaneously with, the yeast synthesis, a part of the sugar is degraded by respiration in accordance with the equation:

$$C_6H_{12}O_6 + 6\ O_2 = 6\ CO_2 + 6\ H_2O$$

Thus, in order to calculate the maximum biological yield, it is necessary to know, among other things, the amount of sugar respired by the yeast cells during the synthetic reaction. This may be determined by measurement of the amount of oxygen consumed during the synthesis in a respirometer, such as that constructed by Sperber (1942). Brahmer reckons with an oxygen consumption of 943 g per 1000 g of dry yeast substance formed (cf. Sperber 1945), corresponding to 884 g glucose. From this, and equations 1 and 2, Brahmer calculates the maximum biological yield in the following manner: 740 g dry yeast substance requires 1000 g glucose, while 0.74×884 g $= 654$ g glucose is simultaneously respired. The total glucose consumption is therefore 1654 g. Hence at the most 447 g dry yeast substance is obtained per 1000 g glucose, corresponding to 471 g per 1000 g saccharose. In this connection Brahmer also mentions the importance of the nitrogen content of the yeast in the assessment of the maximum biological yield. He emphasises, as Fink has also pointed out, that the yeast yield must vary with the protein content of the yeast. If the yeast be regarded as a mixture of protein and carbohydrate and if the CHO residue of the protein is given the formula $C_6H_{10}O_2$ (cf. EFFRONT 1927 and CLAASSEN 1934 a) and the carbohydrate fraction, the polysaccharide, the formula $C_6H_{10}O_5$, it will be seen from the equations

$$2\ C_6H_{12}O_6 = 2\ C_6H_{10}O_2 + 2\ H_2O + 3\ O_2$$

$$2\ C_6H_{12}O_6 = 2\ C_6H_{10}O_5 + H_2O$$

that in the formation of CHO residue of protein, three atoms of oxygen are split off, which then give rise to further combustion of sugar. In the incorporation of carbohydrate in the yeast cell, however, no oxygen is liberated. The higher, therefore, the protein content of the yeast cell is, the lower will be the yield of yeast calculated on the sugar consumption. BRAHMER shows that this difference amounts to 64 % for the limiting cases of yeasts with protein contents of 25 and 70 %, i.e. the yield of yeast with 25 % protein will be 64 % greater than when the product contains 70 % protein. With respect to protein content, however, the maximum biological yields in these two cases are equivalent. This conclusion is somewhat at variance with the results of SPERBER (1945) who emphasises that when the nitrogen supply is inadequate the yeast produces from the sugar, in the place of protein, corresponding amounts of fat and polysaccharide, so that the yeast yield remains practically unchanged. It should perhaps be noted, however, that FINK and BRAHMER worked with nitrogen present in excess, so that the conditions for the yeast synthesis are incomparable in the two cases.

The figure employed by BRAHMER for the calculation of the maximum biological yield, 943 g oxygen per 1000 g dry yeast substance formed, is derived from yeast cultivation experiments in SPERBER's respirometer, which were carried out with raw beet sugar molasses as substrate. Apart from sugar, however, such substrate contains other organic materials which can serve as direct sources of carbon for the growing yeast and of which the oxygen requirements are not accurately known. It may therefore be taken as certain that the actual consumption of oxygen in the synthesis of yeast from pure sugar is different from that reported by BRAHMER. In Chapter II, p. 24—31, yeast cultivation experiments in the respirometer are described, which were carried out with saccharose as the sole carbon source. As the mean value from 16 experiments, the oxygen consumption was 650 l at N. T. P., or 929 g, per 1000 g dry yeast substance formed. (These are the experiments of Table 8, where the RQ values lay between 1.01 and 1.06). The yeast yield was 510 g dry yeast substance per 1000 g saccharose and the yeast had a protein content of 48.5 %. If we accept BRAHMER's value for the stoichiometric yield, 740 g dry yeast substance per 1000 g glucose, corresponding to 779 g per 1000 g saccharose, and if we calculate the amount of saccharose corresponding to 929 g oxygen as 827 g, the maximum biological yield is 474 g dry yeast substance per 1000 g saccharose. On the basis of his theoretical considerations, supported by experimental evidence, BRAHMER has derived a formula for the calculation of the maximum biological yield of yeast from glucose:

$$J = \frac{1}{(p\,(R + 0.55) + 1.11)\,a}$$

where

J = g dry matter obtained per g glucose.

p = weight of newly formed protein per g ash-free dry yeast substance

$$= \frac{N \times 6.25}{100}$$

$R = 1.52 \times O_2$, where O_2 = respiration oxygen per g protein during J g growt
with $RQ = 1.00$

$a = \frac{100 - A}{100}$ where A = percentage ash in dry yeast substance.

If the results of the yeast cultivation experiments I a, I b and I c in Table (Chapter II, p. 26—27) are used to calculate the maximum biological yield wit the aid of BRAHMER's formula and these are compared with the values actuall obtained, the outcome is as shown in Table 1.

Table 1

Calculated and actual yields of yeast in cultivations with saccharose in th respirometer.

Experiment	Calculated maximum yeast yield, g per 1000 g saccharose	Actual yeast yield, g dry substance per 1000 g saccharose	Percentage difference between calculated and actual yield
I a	526	478	9.1
I b	550	524	4.7
I c	539	496	8.0
Mean of I a, I b and I c	538	499	7.2

A corresponding calculation with the experimental results from the 12 yeast cultivation experiments of Table 8, where the RQ lay between 1.01 and 1.06, gave, when the mean values for all 12 experiments were employed, a maximum biological yield of 555 g dry yeast substance per 1000 g saccharose, as against an actual yield of 510 g, i.e. 8.1 % too low.

In view of the experimental difficulties in respirometer yeast cultivations and the many factors influencing the growth of yeast, the agreement between the yields calculated as possible and actually obtained may be considered as reasonable.

PART II

Chapter II. Yeast cultivations in the laboratory

In laboratory yeast cultivations, primarily intended for the study of yeast growth, care must be taken that the substrate can be strongly aerated during the fermentation. Otherwise, as has already been explained, the degradation reactions proceeding simultaneously with the yeast synthesis will lead to the formation of alcohol, so that the synthesis of yeast substance will decrease. Hence we must exclude from the outset the more or less anaerobic cultivations in glass flasks, in which air is not simultaneously blown through the medium.

1. Cultivations in open fermentation vessels

Claassen (1926) used for his laboratory experiments a fermentation vessel of sheet metal with a square cross-section of 15 cm and a height of 60 cm. On the bottom of the vessel lay finely perforated aeration tubes. The vessel was placed in a water thermostat.

Effront (1927) employed a strongly diluted glucose solution of 0.3 % concentration. He gives the fermentation time as 7–8 hours and the total amount of sugar added in each experiment as 150 g. Unfortunately he gives no account of his apparatus.

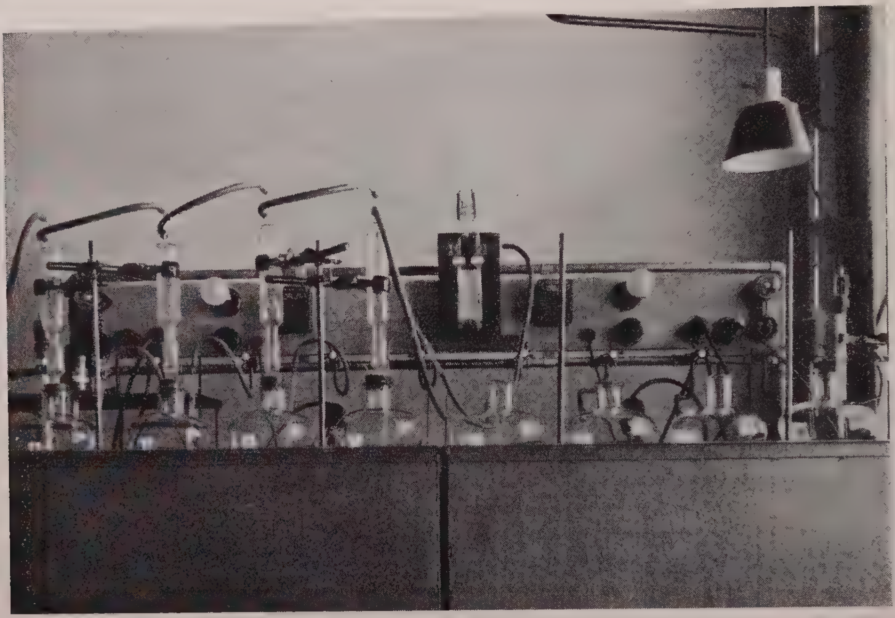
An extremely valuable account of suitable apparatus for laboratory cultivations is given by Fink and co-workers (1938 b) in a series entitled "Beiträge zur biologischen Zellsubstanz-Synthese der Hefe".

The actual cultivation apparatus consists of one or more glass cylinders, each of about 100 cm height and 10 cm internal diameter. These cylinders, which are provided with ground glass flanges, may be connected by means of suitable packing. A tower-like cultivation chamber is thus formed, which may be rendered air-tight by means of a base plate and a lid. The aeration takes place through the base plate, usually through porolith rods. The plate also carries a coil of metal tubing for heating or cooling purposes and a valve for the removal of liquid. The lid bears the gas exit tube and devices for the introduction of nutrient solution and fat for hindering of formation of foam. If the gaseous materials formed during a cultivation are to be investigated, the gases leaving the vessel are passed through an absorption train. In his standard experiments in this apparatus Fink worked with a 1 % sugar solution and a total volume of 7 litres.

Enebo, Lundin and Myrbäck (1941) in their experiments on the cultivation of yeast on whey, used an apparatus similar to that of Fink, but somewhat simplified, since they were concerned only with the determination of the yeast yield and not with the carbon balance. The apparatus consisted of a glass cylinder of 10 cm cross-section and 250 cm height. As an air-distributor a glass filter funnel (Jena, No. 25G3) was inserted in the bottom of the cylinder. The whole apparatus was set up in a thermostated room at about 26° C., so that heating contrivances in the cylinder itself were unnecessary. The introduction of nutrient fluid took place from a reservoir mounted above the cylinder.

Similar laboratory cultivation apparatus, where the fermentation space is in the form of a cylinder, is in general use in fermentation laboratories.

In the present investigation, where several fermentations were carried out simul-



taneously, the vessels employed were Kluyver flasks. These flasks, with a total volume of 3 litres, make it possible to work comfortably with volumes of wort of up to 2000 ml. The inverted conical form of the vessels hinders the formation of troublesome heights of foam, which may in any case readily be suppressed with Turkey red oil. The conical form of the vessels has another advantage in that it ensures a highly effective agitation of the liquid by the air stream and eliminates the possibility of a dead angle which might give rise to sedimentation of the yeast. In the cultivation experiments the usual crop was 20–30 g yeast of 25 % dry weight, which was quite adequate for the necessary determinations.

The air was taken from the laboratory compressed air supply and was cleaned by passage through a cotton-wool filter. The temperature was controlled by standing the vessels in a water bath fitted with a stirrer and a thermoregulator.

As is shown in the accompanying photograph, the apparatus consisted of eight flasks in two groups of four, each group forming a fermentation battery in its own water bath. Each flask had its own air supply, which could be regulated by a finely adjustable tap. The air flow could be measured by the introduction of a flow-meter. The fermentation flasks were fitted with condensers, in order to prevent excessive evaporation.

In all experiments 10 g sugar, or a corresponding amount of molasses, was employed. The nutrient solution was always added in graduated portions during the cultivation. The cultivation temperature was between 21 and 28° C. and, when not otherwise stated, the amount of seed yeast was 5 g of 25 % dry weight per 10 g sugar. The time of cultivation was so chosen that on each occasion fully mature yeast was obtained, i.e. a yeast that no longer budded. This time varied between 12 and 18 hours. In some cases the alcohol formed was estimated by distillation. The water employed was ordinary tap water.

At the end of the cultivation the yeast was immediately worked up in order to prevent any further reactions such as might take place on long standing. Firstly, the total volume of the yeast suspension was measured and aliquots were transferred, for yield estimations, to centrifuge tubes of about 50 ml capacity, or filtered through small Jena glass filters. After the centrifugation or filtration, the samples were washed twice with distilled water and the yeast so obtained was dried at 105° C. On the basis of the original quantity of seed yeast and the total volume, it was then possible to estimate the yield. The greater part of the fermented substrate was spun on a rapid centrifuge. The yeast was then transferred as quantitatively as possible to a suction filter and hereby dried to the normal water content of about 75 %. The spent wort, freed from yeast, was slightly acidified with sulphuric acid and kept in the refrigerator for further analysis.

In the following section two yeast cultivation experiments will be described, which will serve as standard examples of the laboratory cultivations carried out in the present investigation.

Yeast cultivation experiment I in synthetic nutrient medium or in molasses solution

Composition of nutrient substrate:

111.0 g saccharose or a corresponding quantity of molasses dissolved in tap water to a final volume of 1 litre. During the cultivation the following quantities of nutrient substances were added per litre of substrate:

2.8 g $(NH_4)_2SO_4$
7.3 g $(NH_4)_2HPO_4$
0.4 ml 25 % NH_3

If saccharose was employed, the substrate was complemented with further nutrient salts (g per l substrate):

18.0 g K_2SO_4
3.6 g NaCl
0.6 g $CaCl_2$
0.3 g $MgSO_4 \cdot 7\ H_2O$
11.0 g NH_4Cl

Cultivation time: 12 hours.
Seed yeast: 5 g with 25 % dry weight.
Total quantity of sugar used: 10 g.
Total volume: about 725 ml.

At the beginning of the cultivation 600 ml tap water together with 1.0 ml 5 % $(NH_4)_2SO_4$ solution and 3.0 ml 6 % $(NH_4)_2HPO_4$ solution were placed in the Kluyver flask. After the addition of 5 g seed yeast of 25 % dry weight, 20 ml of the diluted sugar nutrient solution was added. In the first three hours 100 litres of air was passed per hour, during the main part of the fermentation 120 litres per hour, and during the last hour 50 litres. The temperature was set at 23° C. and rose during the fermentation to 26° C. The pH was adjusted with dilute sulphuric acid to 5.8 at the commencement of the experiment and fell in the course of the first three hours to 5.0, where it was maintained by the alternate addition of $(NH_4)_2SO_4$ and NH_3. During the last hour the pH rose to 6.2. The nutrient substrate was added, commencing after the second hour, in graduated portions every 15 minutes. The total period of cultivation was 12 hours. At the end of the cultivation the yeast was immediately worked up by the procedure already described on p. 21.

The course of the cultivation experiment is summarised in Table 2.

Table 2

Scheme for yeast cultivation experiment on synthetic substrate under aerobic conditions.

Cultivation time, hours	ml diluted substrate	ml 5 % $(NH_4)_2SO_4$	ml 6 % $(NH_4)_2HPO_4$	ml NH_3 (1 ml 1.7 mg N)	pH	Aeration, litres per hour	Temp. °C.
0	20	1	3	—	5.8	100	23
1	—	—	—	—	5.5	—	—
2	5	1	1	0.5	5.2	—	—
3	7	1	1	—	5.0	—	—
4	8	—	1	0.5	5.0	120	—
5	8	—	1	0.5	5.0	120	24
6	8	1	1	1.0	5.0	120	—
7	10	—	1	—	5.0	120	—
8	10	—	—	1.0	5.0	120	26
9	10	1	1	—	5.0	120	—
10	4	—	1	—	5.0	120	—
11	—	—	—	—	5.6	120	—
12	—	—	—	—	6.2	50	—

In the experiment described above 4.6 g dry yeast substance was obtained from 10 g saccharose. The protein content of the seed yeast was 60.9 % and that of the crop yeast 49.1 %. From the biological viewpoint the yeast was fully mature, i.e. without budding, free from bacteria and other infection. The cells were of an even, average size with clear plasma and with isolated, large vacuoles. The substrate, when freed from yeast, contained no alcohol.

Yest cultivation experiment II in synthetic nutrient medium or in molasses solution

Composition of nutrient substrate:

33.7 g saccharose or a corresponding quantity of molasses dissolved in tap water to a final volume of 1 litre. During the cultivation the following quantities of nutrient substances were added per litre of substrate:

5.0 g $(NH_4)_2SO_4$
8.0 g $(NH_4)_2HPO_4$
0.9 ml 25 % NH_3

If saccharose was employed, the substrate was complemented with further nutrient salts (g per 1 substrate):

5.5 g K_2SO_4
1.1 g NaCl
0.2 g $CaCl_2$
0.07 g $MgSO_4 \cdot 7\ H_2O$
3.3 g NH_4Cl

Cultivation time: 17 hours.
Seed yeast: 5 g with 25 % dry weight.
Total quantity of sugar used: 10 g.
Total volume: about 760 ml.

At the beginning of the cultivation 385 ml tap water, 5 ml 1 % $(NH_4)_2SO_4$ solution, 5 ml 1 % $(NH_4)_2HPO_4$ solution, 5 g 25 % seed yeast and 30 ml of the nutrient solution were placed in the Kluyver flask. In the first three hours 100 litres of air was passed per hour, during the main part of the fermentation 120 litres per hour and during the last hour about 50 litres. The temperature was set at 21° C. and rose during the fermentation to 26° C. The pH was adjusted with dilute sulphuric acid to 5.8 at the commencement of the experiment and fell during the first 5 hours to 5.0. It was then maintained at between 5.0 and 5.5 until the last hour but one. During the final hour the pH rose to 6.5. The substrate was added in portions over 15 hours, at first half-hourly and then quarter-hourly.

The course of the cultivation is summarised in Table 3.

Table 3

Scheme for yeast cultivation experiment II.

Cultivation time, hours	ml diluted molasses	ml 1 % $(NH_4)_2SO_4$	ml 1 % $(NH_4)_2HPO_4$	ml NH_3 (1 ml 1.7 mg N)	pH	Aeration, litres per hour	Temp. °C.
0	30	5	5	—	6.5	100	21
1	—	—	—	—	—	—	
2	9	—	—	—	5.2	—	
3	9	—	1	—	5.0	110	
4	12	1	1	1	4.9	—	
5	12	1	1	1	5.0	—	22
6	14	1	1	3	5.2	115	
7	16	1	1	3	5.2	—	
8	18	2	2	2	5.2	—	23
9	20	2	2	2	5.3	—	
10	20	4	2	1	5.3	115	
11	24	4	2	2	5.3	—	24
12	31	5	2	1	5.4	—	
13	30	5	2	1	5.4	—	26
14	30	3	1	3	5.4	—	
15	15	2	1	3	5.4	—	
16	—				—	50	
17	—				6.5		

In an experiment with molasses as substrate in accordance with the above scheme, 5.14 g dry yeast substance was obtained from 10 g sugar. The protein content of the seed yeast was 59.8 % and that of the crop yeast 48.1 %. The yeast crop was fully mature, without buds, free from bacteria and other infections. The fermented substrate contained no alcohol.

2. Cultivations in respirometers

a) With saccharose as substrate

In following the yeast synthesis in a given cultivation, it is insufficient to consider only the yield. It is also necessary to take into account the carbon dioxide formed, the oxygen consumed and also any alcohol produced in the cultivation. The apparatus described above, however, does not permit the estimation of the carbon dioxide formed or the oxygen consumed during the experiment, since open vessels are employed.

The literature contains several descriptions of apparatus which allows for the determination of carbon dioxide production and oxygen consumption during the biological degradation of sugar. Such apparatus includes various types of respirometers and the well-known Warburg apparatus. The Warburg procedure does not, however, permit a continuous addition of substrate and employs, moreover, concentrations of sugar and yeast quite different from those used in actual yeast cultivations. It is therefore inadequate for the study of yeast growth where our principal concern is with the yield of yeast. The various respirometers, on the other hand, not only make it possible to determine the oxygen and the carbon dioxide, but also permit the continuous introduction of substrate according to a definite scheme.

Wood, Brewer, Michelson and Werkman (1940), who employed closed systems, determined the carbon dioxide evolved by absorption in 40 % KOH and weighed the carbonate formed in the same manner as in elementary analysis. The oxygen consumption was determined by measurement of the reduction of the total gas volume in the respirometer. Fink and Münder (1942) used the standardised yeast cultivation apparatus described by Fink, Lechner and Krebs (1938 b) for the aeration procedure, the carbon dioxide being measured in this case also by absorption in KOH. The oxygen consumption was found by analysis of the air drawn through the apparatus, before and after the experiment, the required value being calculated by difference. In a respirometer constructed by Werthessen (1937), chiefly for animal experiments, a closed system was also employed. The carbon dioxide was absorbed in KOH and measured conductimetrically. The oxygen used during the experiment was replaced electrolytically. Sperber (1942) describes a respirometer which is a combination of the instrument of Wood *et al.* and Werthessen. The yeast cultivation is carried out in a closed system in Kluyver flasks. The carbon dioxide is measured conductimetrically after absorption in NaOH, while the consumed oxygen is replaced electrolytically during the experiment and estimated from the quantity of electricity required for this replacement as measured by an ampere-hour meter.

Fink's investigations were carried out exclusively with *Torula utilis*, whereas Sperber (1945) describes two experiments with *Saccharomyces cerevisiae* and molasses as substrate. Sperber's respirometer was available for the yeast cultivation experiments described in the present work. As will be shown in the account given below, both pure sugar solutions and molasses were employed as nutrient substrates.

The main purpose of these cultivation experiments, apart from the yield determinations, was the estimation of the carbon dioxide produced during the cultivation and of the oxygen consumed, in order that these data might be employed in following the biochemical course of the yeast growth. According to the reaction equations applying to the degradation of sugar in the presence of assimilable nitrogen:

Table 4

Yeast cultivation experiments in the respirometer.

Nutrient substrate: Saccharose.
Seed yeast: Z III (cf. Chapter II, β).
Cultivation time: 11 hours.
Temperature: 24 °C.
Initial volume: 270 ml.
Final volume: 320 ml (about).

Cultivation time, hours	Addition of substrate												Carbon dioxide evolved, ml			Oxygen consumed, ml		
	mg sugar as saccharose			mg nitrogen as $(NH_4)_2SO_4$			mg nitrogen as $(NH_4)_2HPO_4$			mg nitrogen as NH_3								
	I a	I b	I c	I a	I b	I c	I a	I b	I c	I a	I b	I c	I a	I b	I c	I a	I b	I c
0	320	320	320	8.2	8.2	8.2	12.5	12.5	12.5	—	—	—	—	—	—	—	—	—
1—3	352	352	352	9.0	9.0	9.0	8.5	8.5	8.5	3.4	3.4	3.4	196	193	213	187	182	176
3—5	446	457	446	13.3	13.7	13.3	8.4	8.6	8.4	—	—	—	177	191	183	175	181	169
5—7	618	613	620	19.8	19.5	19.8	4.5	4.4	4.5	3.4	3.4	3.4	136	206	149	138	193	159
7—9	635	625	628	9.7	9.5	9.6	—	—	—	5.4	5.3	5.4	275	173	243	239	179	206
9—10	134	132	134	2.0	2.0	2.0	—	—	—	—	—	—	68	65	75	73	49	78
10—11	—	—	—	—	—	—	—	—	—	—	—	—	25	—	15	28	30	32
	2505	2499	2500	62.0	61.9	61.9	33.9	34.0	33.9	12.2	12.1	12.2	877	828	878	840	814	820

Ta

Yields, chemical composition of seed yeast and crop yeast, carbon dioxide evolut

No. of experiment	Amount of saccharose used, g	Yeast yield, less seed yeast, in g dry substance per 2.5 g sugar	Yield of dry yeast substance in g per 100 g saccharose	Seed yea			
				% N calc. on dry weight	% protein calc. on dry weight	Amount used in mg dry weight	Amou of N us mg
I a	2.5	1.19	47.8	9.5	59.4	626	59.5
I b	2.5	1.31	52.4	9.5	59.4	625	59.4
I c	2.5	1.24	49.6	9.5	59.4	624	59.3

1. $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$

2. $C_6H_{12}O_6 + NH_3 \rightarrow \text{yeast substance} + CO_2$

the ratio of carbon dioxide produced to oxygen consumed during the biological protein synthesis, i.e. the respiratory quotient, must be only slightly above unity. If the quotient is in fact significantly greater than unity, a large part of the sugar breakdown must proceed after the equation

3. $C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$

i.e. the reaction leads to the formation of alcohol, in which case the RQ will be greater than 1, becoming infinity in the limiting case where only alcohol is formed. The carbon dioxide formed according to equation (2) will be small in comparision to that formed according to (1), but is sufficient to account for the fact that the RQ lies somewhat above unity.

In the following three yeast cultivation experiments, I a, I b and I c, carried out in parallel, the course of the cultivation was followed in detail, in relation both to the addition of substrate and to the amounts of carbon dioxide formed and oxygen consumed. The cultivation extended over 11 hours, during which a total of 2.5 g saccharose was added. In order that the principle of the continuous process might be followed with respect to the yeast formed during the cultivation, the additions of sugar substrate were made in three main groups. The experiment commenced with 0.32 g sugar and 0.626 g seed yeast (calculated as dry weight) in a total volume of 270 ml. At the end of the first hour the additions were commenced and 0.35 g sugar was added during the subsequent two hours. In the following two-hour periods additions of 0.45 g, 0.62 g and 0.64 g sugar were made, and in the ninth and tenth hours, constituting the final addition period, 0.1 g. The eleventh hour served for the maturing of the yeast, i.e. for the assimilation of any unused substrate.

The nitrogen was added as ammonium salts to the wort in definite amounts together with the sugar solution. The other elements necessary for the development of the yeast, such as P, K, Na, Mg, Ca and Fe, were placed in the flask at the beginning in the form of inorganic salts. The water employed in the experiment was ordinary tap water. The cultivation temperature was kept constant at 26° C. and the pH was maintained within the range 4.5–5.5.

oxygen consumption in yeast cultivation on saccharose in the respirometer.

	Crop yeast				CO_2 evolved, litres per kg dry yeast substance formed	O_2 consumed, litres per kg dry yeast substance formed	RQ
nount rotein d, mg	% N calc. on dry weight	% protein calc. on dry weight	mg N	mg protein			
72	8.03	50.2	146	913	737	706	1.04
71	7.43	46.4	144	900	632	621	1.02
71	7.66	47.9	143	894	708	661	1.07

Table 6

Nitrogen balance sheet for yeast cultivations on saccharose in the respirometer.

No. of experiment	mg nitrogen added in				Total nitrogen added, mg	mg nitrogen recovered in		Total nitrogen recovered, mg	Percentage nitrogen recovered
	seed yeast	$(NH_4)_2SO_4$	$(NH_4)_2HPO_4$	NH_3		crop yeast	residual wort		
I a	59.5	62.0	33.9	12.2	167.6	146	12.6	158.6	94.6
I b	59.4	61.9	34.0	12.1	167.4	144	15.8	159.8	95.4
I c	59.3	61.9	33.9	12.2	167.3	143	13.1	156.1	93.3

Table 7

Carbon balance sheet for yeast cultivations on saccharose in the respirometer.

The carbon content of the yeast was not determined, but was assumed to be 46 % on the basis of the elementary analyses made by BRAHMER (1932).

No. of experiment	mg carbon added in		Total carbon added, mg	mg carbon recovered in		Total carbon recovered, mg	Percentage carbon recovered
	seed yeast	sugar		crop yeast	CO_2		
I a	288	1052	1340	835	473	1308	97.6
I b	288	1049	1337	890	447	1337	100.0
I c	287	1050	1337	857	474	1331	99.6

The pH cannot be determined and regulated in the course of the cultivation in a completely satisfactory manner, since the Kluyver flasks have no extra side openings to permit the introduction of measuring electrodes. It is, on the other hand, impracticable to open the flask for pH measurements, since this would render impossible the continuous estimation of carbon dioxide and oxygen. For this reason SPERBER's technique (1945) was adopted, of carrying out yeast cultivations as control experiments, under precisely the same conditions as the main experiments, in which only the pH was measured and not the carbon dioxide and oxygen. At the same time the pH was maintained between 4.5 and 5.5 by the addition of sodium carbonate. The quantities of carbonate found necessary in the control experiment were then added together with the nutrient solution in the main experiment. The added carbonate was taken into account in the evaluation of the carbon dioxide formed during the cultivation. This procedure proved to be quite satisfactory, since the pH of the medium at the conclusion of the experiment usually lay between 5.3 and 5.5.

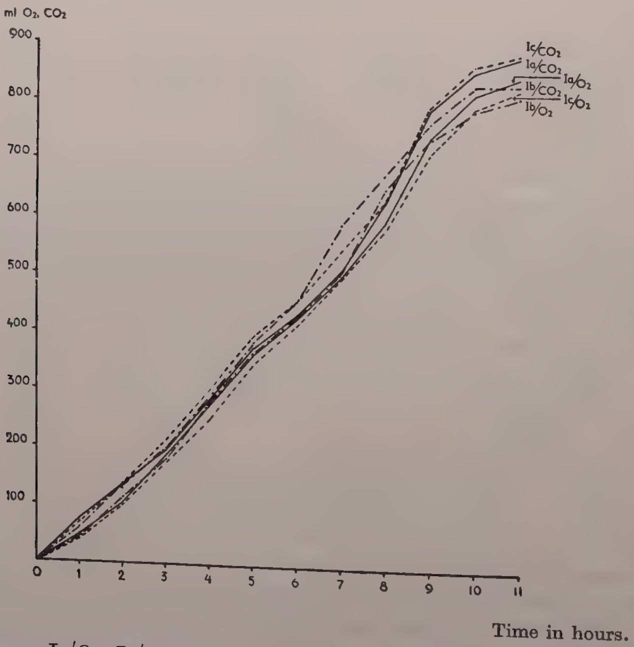
The course of the cultivation experiments with regard to the amounts of substrate, carbon dioxide produced and oxygen consumed, is shown in Table 4.

In Table 5 are shown the yields of yeast, some chemical analyses of the seed yeast and of the crop yeast, the amounts of carbon dioxide developed and of oxygen consumed in yeast cultivations with saccharose in the respirometer.

On the basis of the chemical analyses and of the quantities of carbon dioxide evolved and of oxygen consumed, it is possible to construct nitrogen and carbon balance sheets for the yeast cultivations. These are shown in Tables 6 and 7.

It will be seen from the last columns of the above tables that the cultivations proceed quite satisfactorily throughout with respect to the amounts of N and C consumed, i.e. the added nitrogen and carbon are almost completely recovered in the reaction products.

In Graph 1 the amounts of carbon dioxide formed and of oxygen consumed in the cultivations are plotted as functions of time.



Graph 1. ——— Ia/O_2 Ia/CO_2. —·—·— Ib/O_2 Ib/CO_2. - - - - - - - Ic/O_2 Ic/CO_2
Carbon dioxide evolution and oxygen consumption in respirometer yeast cultivations with saccharose as substrate.

From the carbon dioxide and oxygen values after the tenth hour, the values of the RQ are calculated for curve a as 1.04, for b as 1.02 and for c as 1.07. The close proximity of the O_2 and CO_2 curves shows that the RQ was favourable for the synthesis of yeast substance throughout the experiments, i.e. it approximated to unity, so that no alcohol was formed in the sugar degradation.

Table 8 contains a summary of a series of yeast cultivation experiments, all carried out with saccharose in the respirometer. Common to all experiments was the time of cultivation, 11 hours, and the seed yeast, which was of the stage Z III throughout. The data in the table are as follows:

Column	1	amount of sugar used in g.
»	2	amount of seed yeast, expressed as g dry weight.
»	3	amount of nitrogen used in g, comprising nitrogen of seed yeast and of added NH_3, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$.
»	4	total amount of carbon used in g, comprising carbon of seed yeast and of sugar.
»	5	yields of dry yeast substance in g, calculated for 100 g saccharose.
»	6	oxygen consumed in litres at N. T. P. per 1000 g of dry yeast substance formed.
»	7	carbon dioxide evolved in litres at N. T. P. per 1000 g of dry yeast substance formed.
»	8	g of nitrogen recovered, calculated from the nitrogen content of the crop yeast and the residual nitrogen of the fermented, yeast-free wort.
»	9	g of carbon recovered, calculated from the carbon content of the crop yeast and the carbon dioxide evolved.
»	10	recovered nitrogen as percentage of that added.
	11	recovered carbon as percentage of that added.
»	12	respiratory quotient after 10 hours, i.e. at the end of the addition of substrate.

On the basis of the experimental results in Table 8 several conclusions may be drawn with regard to the course of the yeast synthesis:

1. The amount of seed yeast employed is of great importance for the yield. Thus, the yield is about 39 g dry weight per 100 g saccharose when about 0.3 g dry weight of seed yeast is employed per 2.5 g total added sugar. Amounts of seed yeast corresponding to 0.5 g and 0.6 g dry weight increase the yield to 48.6 g and 50 g dry yeast substance respectively. A further increase to 0.75–0.87 g of seed yeast augments the yield to 53.5 g dry weight per 100 g saccharose, whereas the yield appears to fall off with further increases in the seed yeast.
2. The importance of the amount of seed is also reflected in the amounts of oxygen consumed. For the formation of 1000 g dry yeast substance the consumption of oxygen increases with decreasing amounts of seed yeast. Thus, with about 0.3 g dry weight of seed yeast per 2.5 g saccharose over 800 l oxygen is used in the formation of 1000 g dry yeast substance, while this figure is reduced to about 650 l for 0.6–0.9 g dry weight of seed yeast.
3. The respiratory quotient RQ, calculated from the carbon dioxide production and the oxygen consumption, is related to the yield of yeast, being greatest for the lowest yields. The RQ was 1.11 for a yield of 37 g dry substance per 100 g

Table 8

Yeast cultivation experiments with saccharose in the respirometer.

1	2	3	4	5	6	7	8	9	10	11	12
Sugar used, g	Seed yeast, calc. as dry weight, g	Total nitrogen used, g	Total carbon used, g	Yeast yield, g dry substance per 100 g saccharose	O_2 consumed per 1000 g dry yeast substance formed, litres	CO_2 evolved per 1000 g dry yeast substance formed, litres	Total nitrogen recovered, g	Total carbon recovered, g	Percentage of nitrogen recovered	Percentage of carbon recovered	RQ
2.50	0.311	0.137	1.193	37.0	907	1011	0.135	1.073	98.5	90.0	1.11
2.50	0.312	0.137	1.194	37.9	909	988	0.132	1.085	96.4	90.9	1.09
2.49	0.374	0.143	1.218	38.8	890	997	0.132	1.136	92.3	93.3	1.12
2.50	0.375	0.143	1.223	42.6	818	912	0.141	1.186	98.6	97.0	1.11
2.51	0.500	0.162	1.284	48.8	751	785	0.160	1.308	98.8	101.9	1.05
2.49	0.500	0.155	1.276	48.6	678	708	0.142	1.249	91.6	97.9	1.04
2.51	0.622	0.174	1.340	51.7	633	657	0.169	1.343	97.1	100.2	1.04
2.50	0.622	0.167	1.336	48.9	698	737	0.161	1.335	96.4	99.9	1.06
2.50	0.622	—	1.336	51.1	615	675	—	1.340	—	100.3	1.10
2.50	0.623	0.167	1.337	52.1	660	673	0.162	1.358	97.0	101.6	1.02
2.50	0.625	0.174	1.338	52.0	642	665	0.163	1.351	93.7	100.9	1.04
2.51	0.625	0.167	1.342	46.6	718	791	0.163	1.325	97.6	98.7	1.10
2.50	0.626	0.167	1.338	49.0	705	731	0.166	1.334	99.4	99.7	1.04
2.52	0.750	0.187	1.403	53.8	596	631	0.179	1.429	95.7	101.9	1.06
2.50	0.750	0.179	1.395	49.3	645	693	0.172	1.372	96.1	98.4	1.07
2.49	0.875	0.190	1.449	53.4	576	579	0.181	1.434	95.3	99.0	1.01
2.50	1.120	0.214	1.565	52.4	593	607	0.209	1.546	97.7	98.8	1.02
2.50	1.120	—	1.565	49.8	662	667	—	1.536	—	98.1	1.01
2.50	1.495	—	1.738	51.8	608	617	—	1.714	—	98.7	1.01

saccharose, and 1.04 for a mean yield of 52 g dry substance per 100 g saccharose. Hence the determination of RQ during a cultivation provides a valuable means of following the efficiency of the yeast synthesis.

b) With molasses as substrate

As has already been mentioned, SPERBER reported in 1945 two respirometer experiments with *Saccharomyces cerevisiae* where molasses was employed as substrate. In one experiment he used a beet sugar molasses and obtained 54.5 g dry yeast substance per 100 g polarising sugar. In the second experiment, working with a refined molasses, he obtained 43.5 g dry yeast substance per 100 g polarising sugar. In the cultivation with beet molasses 680 ml oxygen was used in the formation of 1 g dry yeast substance, whereas in the corresponding experiment with refined molasses 870 ml was required. The time of cultivation was 11 hours and the temperature 28° C. Further details of the addition of the substrate during the cultivation and of the oxygen consumed and carbon dioxide evolved at different stages were not reported, so that it is difficult to form an accurate picture of the cultivation process. From the nitrogen and carbon balance sheets, however, it is clear that the added quantities of these elements were very satisfactorily employed.

In the experiments described below, it was therefore our first task to confirm the results obtained by SPERBER. The apparatus was identical with that employed in the experiments with saccharose.

The molasses used was a beet sugar molasses from the campaign of 1944. Its characteristics were as follows: fermentable sugar 48 %, dry weight corresponding to "77° Balling", total nitrogen calculated on dry weight 2.7 %, ash content, as carbonate ash, 9.5 %. The cultivation was carried out in the same manner as with saccharose. The time of cultivation was 11 hours, the temperature 24° C. and the seed yeast was Z III. The molasses was first diluted with water and sterilised for one hour at 100° C. in steam. After dilution to 1 : 2, nutrient substrate was made of this solution together with ammonium sulphate, diammonium phosphate and ammonia and the necessary quantity of sodium carbonate for pH regulation as determined in the control experiment. A separate solution was prepared for each two-hour period. At the beginning of the experiment 250 ml tap water, 1 or 2 ml diluted molasses solution corresponding to 0.160 g or 0.320 g sugar respectively, and 0.5 g dry weight of seed yeast were placed in the Kluyver flask. The aeration was 120 l per hour.

The course of the experiment with regard to additions of substrate, oxygen consumption and carbon dioxide evolution is shown in Table 9. This and the following tables are analogous to the corresponding tables for the respirometer cultivations with saccharose.

Table 10 gives the data concerning the yeast yields, the chemical composition of the seed and crop yeasts and the quantities of oxygen consumed and carbon dioxide evolved in litres at N. T. P. per kg of dry yeast substance formed.

From the analytical values in Table 10, and assuming that the carbon contants of the seed and crop yeasts were 46 %, the nitrogen and carbon balance sheets shown in Tables 11 and 12 may be drawn up.

In the drawing up of the carbon balance, only the carbon of the seed yeast and that of the fermentable sugar are taken into account.

Ta

Yeast cultivations

Substrate: molasses
Seed yeast: Z III (cf. Chapter II,
Cultivation time: 11 hours

Cultivation time in hours	Addition in substrate											
	mg sugar in molasses				mg nitrogen as $(NH_4)_2SO_4$				mg nitrogen as $(NH_4)_2HPO_4$			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
0	320	160	160	320	3.8	3.8	3.8	3.8	12.2	12.2	12.2	12.
1–3	347	352	352	352	3.9	3.9	3.9	3.9	8.4	8.4	8.4	8.
3–5	440	448	445	445	7.6	7.8	7.8	7.8	8.3	8.4	8.4	8.
5–7	620	624	620	620	11.6	11.7	11.6	11.6	4.1	4.4	4.2	4.
7–9	624	624	637	631	—	—	—	—	—	—	—	—
9–10	143	134	136	136	—	—	—	—	—	—	—	—
10–11	—	—	—	—	—	—	—	—	—	—	—	—
	2494	2342	2350	2504	26.9	27.2	27.1	27.1	33.0	33.4	33.2	33.

Ta

Yields, chemical composition of seed yeast and crop yeast, and carbon dioxide evo

No. of experiment	Amount of molasses sugar used g	Yeast yield, less seed yeast, in g dry substance	Yield of dry yeast substance in g per 100 g sugar	Seed yeast				
				% N calc. on dry weight	% protein calc. on dry weight	Amount used in mg dry weight	Amount of N used, mg	Amou of prote used mg
I	2.49	1.29	51.8	9.46	59.12	500	47.3	296
II	2.34	1.15	49.1	9.40	58.75	500	47.0	294
III	2.35	1.17	49.8	9.86	61.63	500	49.3	308
IV	2.50	1.22	48.8	9.86	61.63	500	49.3	308

Several useful conclusions may be drawn by comparison of the cultivations on molasses with those on pure saccharose.

It will first be observed that the oxygen consumption of the growing yeast is appreciably greater in relation to the added sugar in the molasses cultivation than in the corresponding experiment with saccharose. Thus, when molasses is employed, about 800 l oxygen is consumed in the formation of 1 kg dry yeast substance, the figure in the corresponding saccharose cultivation being about 650 l. Among other things, this is attributable to the fact that the molasses contains substances other than sugar that are attacked in the metabolic processes, without, however, taking respirometer.

erature: 24° C.
l volume: 250 ml
. volume: about 300 ml

	mg nitrogen as NH_3			CO_2 evolved ml				O_2 consumed ml			
	II	III	IV	I	II	III	IV	I	II	III	IV
—	—	—	—	—	—	—	—	—	—	—	—
.4	3.5	3.5	3.5	200	150	135	192	172	141	133	168
—	—	—	—	149	167	185	198	152	164	159	163
.4	3.4	3.4	3.4	248	250	222	240	236	225	216	240
.3	5.3	5.4	5.4	362	280	324	305	311	277	309	308
—	—	—		100	85	84	66	107	84	78	82
—	—	—		40	38	25	52	47	45	32	44
.1	12.2	12.3	12.3	1099	970	975	1053	1025	936	927	1005

and oxygen consumption in yeast cultivations on molasses in the respirometer.

Crop yeast				CO_2 evolved, litres per kg dry yeast substance formed	O_2 consumed, litres per kg dry yeast substance formed	RQ
N calc. n dry eight	% protein calc. on dry weight	mg N	mg protein			
7.99	49.9	143.0	894	852	795	1.07
8.15	50.9	134.5	838	843	814	1.04
8.06	50.4	134.6	841	833	792	1.05
7.74	48.4	133.1	832	863	824	1.05

part to the same extent as sugar in the yeast synthesis. Among these substances are the amino acids of the molasses. A pure chemical oxidation may also be of importance. A further valuable conclusion may be arrived at from the two carbon balance sheets. In the saccharose experiment practically 100 % of the added sugar is recovered, while in the molasses experiment more carbon is recovered than has been added in the form of yeast and sugar. This excess obviously derives from the molasses, i.e. from substances other than sugar which are degraded simultaneously with the latter.

It is clear from Tables 5 and 10 that the yield of yeast is dependent upon the

Table 11

Nitrogen balance sheet for yeast cultivations with molasses in the respirometer.

No. of experiment	mg nitrogen added					mg total added N	mg nitrogen recovered in		mg total recovered N	Percentage recovery of added N
	seed yeast	molasses	$(NH_4)_2SO_4$	$(NH_4)_2HPO_4$	NH_3		yeast crop	residual wort		
I	47.3	107.1	26.9	33.0	12.1	226.4	143.0	94.0	237.0	105
II	47.0	101.0	27.2	33.4	12.2	220.8	134.5	87.0	221.5	100
III	49.3	101.0	27.1	33.2	12.3	222.9	134.6	88.3	222.9	100
IV	49.3	108.0	27.1	33.2	12.3	229.9	133.1	92.5	225.6	98

Table 12

Carbon balance sheet for yeast cultivations with molasses in the respirometer.

The carbon content of the seed and crop yeast is taken as 46 %.

No. of experiment	mg carbon added in		mg total added C	mg C recovered in		mg total recovered C	Percentage recovery of added C
	seed yeast	sugar of molasses		yeast crop	CO_2		
I	230	1046	1276	823	593	1416	111
II	230	983	1213	759	523	1282	106
III	230	987	1217	769	526	1295	106
IV	230	1050	1280	791	568	1359	106

quantity of oxygen consumed, lower oxygen uptakes corresponding to higher yields. This is direct evidence in support of the proposition that the yeast synthesis should be carried out in such a manner that the ratio $CO_2 : O_2$ lies as close as possible to unity. If this is the case, the addition of substrate must be so regulated that the yeast is provided with only so much sugar as it can employ in the formation of cell substance.

3. The effect of substrate concentration

In the following account of cultivation experiments, the term "substrate concentration" will be employed to denote the ratio of the weight of molasses (g) used to the final volume (ml) of the fermentation mixture. The various concentrations were obtained by varying the initial volume, the concentration of the added nutrient solution, i.e. the stock wort, being kept constant.

The literature contains very different reports concerning the concentration of nutrients employed in yeast cultivation experiments with intermittent addition of substrate, without taking into account their significance with respect to the yield of yeast. Thus, EFFRONT uses 0.3 % sugar solution, while CLAASSEN describes cultivation experiments in which he carries out the fermentation in 2–3 % sugar solution. In an investigation on the metabolic balance, respiration and gas exchange of yeast cells in the aeration process, CLAASSEN (1935) makes use of a wort with 1.3 %

Table 13

Yeast cultivations with different substrate concentrations.

Substrate: molasses (pol. 47.2 %).
Cultivation process: cf. p. 21.
Seed yeast: 5 g Z III with 25 % dry weight.

Initial volume, ml	Final volume, ml	Substrate concentration	Yield of yeast, less seed yeast, g dry substance per 100 g sugar
300	395	1 : 19	46.7
400	495	1 : 23	50.0
500	600	1 : 28	52.0
600	680	1 : 32	54.0

Table 14

Yeast cultivations with different substrate concentrations.

Substrate: molasses (pol. 50 %).
Cultivation process: cf. p. 21.
Seed yeast: 5 g Z III with 25 % dry weight.

Initial volume, ml	Final volume, ml	Substrate concentration	Yield of yeast, less seed yeast, g dry substance per 100 g sugar
300	405	1 : 20	48.9
400	505	1 : 25	50.8
500	615	1 : 31	52.8
600	710	1 : 36	53.4
700	815	1 : 41	53.8
800	910	1 : 46	53.9
900	980	1 : 49	54.5
1000	1095	1 : 55	54.5

sugar. In their laboratory experiments with molasses as substrate, Braun and Pfundt (1936) worked with a molasses concentration of about 3.33 %.

In Tables 13 and 14 a summary is given of some of our laboratory yeast cultivations in which different substrate concentrations were utilised.

In a subsequent experiment the substrate was further diluted. The summary of the results in Table 14 shows that an optimum dilution is reached, the surpassing of which has no effect upon the yield of yeast.

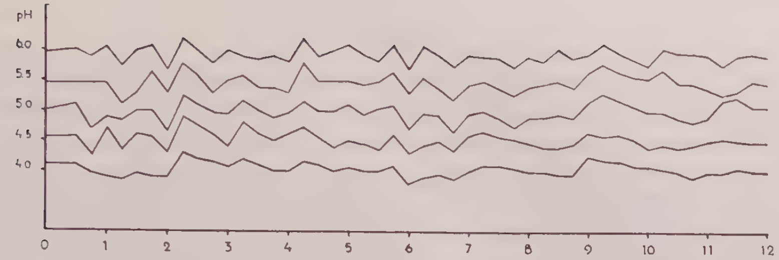
These experiments, the results of which were established in repeated yeast cultivations, show quite clearly that the substrate concentration must be taken into account in the judgment of the yield from a cultivation: only those cultivations are comparable in which the concentrations of substrate are identical or low enough to provide optimal dilution.

4. The effect of pH

As a biological process, the growth of yeast should be highly dependent upon the hydrogen ion concentration of the nutrient substrate BOAS (1936), RIPPEL (1936) and HARTELIUS (1937). Most reports on this matter in the literature are concerned with the changes in pH in more or less anaerobic fermentations, on synthetic substrates or malt or molasses worts. LUERS (1914) showed that yeast in sugar-salt solution reduced the pH of the medium from 5.47 to 2.37 in the course of 160 hours. Similar findings were reported by BOAS (1919). VAN LAER (1922) established a pH optimum of 6.0 for the proliferation of yeast. He also succeeded in demonstrating that this optimum was different for different species.

ELION (1928) found that some baker's yeasts developed better in a neutral than in an acid medium, while Delft yeast behaved in the opposite sense. He also showed that the pH shift in synthetic media was greater than in the so-called beer wort, which contains considerable amounts of buffer substances. SCHWARZ and KAUTZMANN (1931) described experiments with a cultivated top yeast in malt wort and synthetic medium with and without addition of various buffer substances. Over the pH range 3.5–6.5 the yeast crop in malt wort was almost independent of pH, while in synthetic media over the same range a small increase in yield was found on the alkaline side. In malt wort with the addition of phosphate buffer the pH shift was very slight, while citrate buffer allowed the pH to be kept constant. In synthetic media, on the other hand, neither phosphate nor citrate buffer maintained a constant pH. THORNE (1933) reported experiments with Froberg yeast in which he studied the effect of pH upon the growth of yeast and upon the fermentation in synthetic media. He found that the optimum pH of the medium was 4 for yeast growth and 6 for the fermentation. After 8 days the pH in both cases had fallen to 3. He also demonstrated that the final pH was dependent upon the buffering of the substrate. HJORT-HANSEN (1939) studied the growth of yeast, including *Saccharomyces cerevisiae* in synthetic medium at constant pH. As buffer he made use of a mixture of organic acids, lactic, malic, citric, maleic and succinic acids, by means of which he covered a pH range of from 2.5 to 6.0. For the yeast employed he found a pH optimum of 4.8. During the growth of the yeast the pH shifted towards the acid side. No inhibiting effect of the buffers upon the growth was detected.

No conclusions may be drawn from the above investigations concerning the significance of pH for the growth of yeast under aerobic conditions and with continual or intermittent additions of the nutrient medium. Under these circumstances the pH also shifts towards the acid side, but this shift is slowed down by the addition of the nutrient substances ammonium sulphate, diammonium phosphate and ammonia. These additions make it possible to maintain the pH within certain limits, usually in the region of 5. Some experiments were therefore made in order to study in greater detail the influence of pH upon yeast growth under strongly aerobic conditions. Molasses was first employed as nutrient medium. The cultivation time was 12 hours and the pH was kept as constant as possible at 4.0, 4.5, 5.0, 5.5 and 6.0 by the addition of 2 N–NaOH and 2 N–H_2SO_4 during the whole period of the fermentation. The pH was measured potentiometrically every quarter of an hour and adjusted when necessary. In Graph 2 the pH is plotted as a function of fermentation time. In other respects the experiment was performed as the standard experiment described on p. 21. The findings are summarised in Table 15.



Graph 2. Variation in pH in yeast cultivations with molasses as substrate.

Table 15

Effect of pH upon yeast growth.

Nutrient medium: molasses (pol. 4 7%).
Cultivation process: cf. p. 21.
Seed yeast: 5 g Z III with 25 % dry weight.

Initial pH	Yield of yeast, less seed yeast, g dry substance per 100 g sugar	Protein content of yeast crop, %	Volume percentage of alcohol in fermented wort	Final pH
4.12	58.3	45.8	0.15	4.00
4.55	57.5	45.8	0.16	4.50
5.00	57.8	46.3	—	5.08
5.45	56.0	45.0	0.14	5.48
5.95	57.1	46.5	—	5.95

The table shows that the yeast growth is practically independent of the pH over the pH range 4–6. The somewhat lower yields at pH 5.5 and 6 may be attributed to a slight infection of *Diplococci* and sporadic rod bacteria, while the cultivations at pH 4, 4.5 and 5 were practically free from infection. It is well known that the danger of infection is always greater at higher pH values.

In view of the great difficulty in keeping the pH constant during the whole course of the fermentation, which will be obvious from Graph 2, another experiment was made in which the nutrient medium was strongly buffered in the manner adopted by HJORTH-HANSEN (loc. cit.), namely by an addition of a mixture of malic, citric and succinic acids. The wort was made 0.12 N with respect to these acids and the required pH was obtained by the addition of NaOH. The pH values employed were 4, 4.5, 5.5 and 6.

The experiment was carried out in a manner identical with the preceding one. The results are summarised in Table 16.

The result of this experiment was surprising in that the yeast growth decreased greatly with increasing pH. A further series of experiments were performed at constant pH with synthetic nutrient medium. This contained per 100 ml: 11.1 g saccharose, 1.8 g K_2SO_4, 0.35 g NaCl, 0.05 g $CaCl_2$, 0.025 g $MgSO_4$, 4.0 g NH_4Cl, 0.7 g $(NH_4)_2HPO_4$, 0.3 g $(NH_4)_2SO_4$, 0.81 g malic acid, 0.84 g citric acid and 0.71 g succinic acid. The pH was adjusted to 4, 4.5, 5, 5.5 and 6 by addition of 2 N–NaOH. Of this solution 90 ml, corresponding to 10 g sugar, was used in each

Table 16

Nutrient medium: molasses (pol. 47 %) with organic acid mixture as buffer.
Cultivation process: cf. p. 21.
Seed yeast: 5 g Z III with 25 % dry weight.

Initial pH	Yield of yeast, less seed yeast, g dry weight per 100 g sugar	Protein content of yeast crop, %	Volume percentage alcohol in fermented wort	Final pH
4.00	58.7	43.1	0.16	4.10
4.48	56.7	43.0	0.19	4.60
4.95	54.5	43.3	0.22	5.05
5.50	49.2	43.3	0.25	5.55
5.95	45.4	43.6	0.28	5.75

Table 17

Nutrient medium: synthetic substrate with organic acid buffer.
Cultivation process: cf. p. 21.
Seed yeast: 5 g Z III with 25 % dry weight.

Initial pH	Yield of yeast, less seed yeast, g dry weight per 100 g sugar	Final pH
4.00	44.7	3.65
4.50	41.3	4.30
4.95	33.3	4.80
5.50	22.6	5.32
5.98	21.4	5.60

experiment. The pH was determined potentiometrically during the experiment at hourly intervals. The results are shown in Table 17.

The results indicate that, when the pH is kept constant by organic buffers during this cultivation, the growth of yeast in a synthetic medium is highly dependent upon the pH, falling off with increasing pH. From the results of the three series of experiments:

1. Molasses medium without extra buffer and with pH regulation by NaOH and H_2SO_4, 2. Molasses medium with acid buffer, and 3. Synthetic medium with acid buffer, it may be concluded:

1. The growth of yeast in molasses solution under strongly aerobic conditions is practically independent of pH over the range 4.0–6.0.

2. The growth of yeast in molasses solution and in a synthetic medium under strongly aerobic conditions and with addition of malic, citric and succinic acids as extra buffer materials, falls with increasing pH over the range 4.0–6.0.

The inhibition of yeast growth under aerobic conditions in these latter experiments must hence probably be ascribed to the effect of the buffer substances malic, citric and succinic acids. There is a marked difference in the effects of these organic acids when employed as buffers under aerobic and anaerobic conditions, since in the present aerobic experiments they appear to have a toxic effect upon the yeast, which was not found by HJORT-HANSEN in his anaerobic experiments.

5. The effect of aeration

Several references have already been made to the aeration of the fermenting wort. This aeration has three main purposes, namely: 1. Removal from the wort of carbon dioxide formed, 2. Introduction of oxygen for the respiration of the growing yeast and 3. Stirring of the wort.

Accounts of very detailed investigations, together with references to the previous literature on the effect of carbon dioxide upon the growth of microorganisms, are to be found in the work of BRANDT (1945). This author was able to show that the growth of yeast was slowed down by increasing carbon dioxide concentration in the medium. When the gaseous phase contained 50 % carbon dioxide, the growth over 10 hours was 33 % lower than in the control experiment. BRANDT worked with flat culture flasks through which, after the substrate had been inoculated with a known quantity of seed yeast, a known amount of an air-carbon dioxide mixture was passed. By the repeated passage of new gas mixture at intervals during the experiment, the required concentration of carbon dioxide was maintained. In these determinations considerably higher concentrations of carbon dioxide were employed than have to be reckoned with in aerobic yeast cultivations. As a consequence of the large quantities of air blown through the fermenting wort, such a large excess of air is present that the carbon dioxide concentration is only insignificantly higher than that in the atmosphere and is therefore quite innocuous to the growth of the yeast. NORMANDER, of the Svenska Jästfabriks AB, in an unpublished series of yeast cultivation experiments employing the continuous addition procedure on the semi-industrial scale, showed that the addition of 20 % extra carbon dioxide to the air blown through the wort, was without injurious effect on the yeast growth. Only when a mixture of 80 % carbon dioxide and 20 % oxygen was employed did an inhibition of the growth appear, the formation of alcohol being simultaneously increased. The aeration also serves to provide oxygen for the respiratory processes of the growing yeast cells. From the respirometer cultivation experiments described in Chapter II, we know that for the formation of 1 kg dry yeast substance about 800 litres of oxygen is necessary. The large quantities of air employed in industrial yeast production ensure, with a wide margin, the necessary amount of oxygen.

Finally, it is necessary to mention the agitation of the wort brought about by its aeration. By this means the yeast cells are continually brought into contact with fresh nutrient medium, so that constant conditions are provided for the metabolic processes.

The cultivation apparatus employed and the addition procedures adopted in the present investigation fulfilled these three main demands on the aeration.

6. The effect of the seed yeast

When it is desired to compare the findings in different yeast cultivation experiments, it is essential that the same experimental conditions shall have been employed throughout and that equivalent seed yeasts shall have been used. These requirements, as far as the seed yeast is concerned, are extremely difficult to fulfil since yeast, as a living material, differs in composition and properties according to its metabolic history.

In some cases accounts in the literature give details concerning the seed yeast used in cultivation experiments. CLAASSEN (1926), in his monograph "Molasses as raw material for the preparation of yeast by the aeration procedure", states that he employed "the normal seed yeast of the factory, which did not contain detectable amounts of 'Kahmhefe' ". On another occasion he describes his seed yeast as "commercial yeast". In a paper on the processes involved in the synthesis of the yeast substance, CLAASSEN (1934 a) remarks that "the seed yeast was cultivated in a small yield with addition of malt sprouts". In his work "On the metabolic balance, respiration and gas exchange of yeast cells in the aeration procedure", CLAASSEN (1935) speaks of seed yeast "with vigorous growth". THORNE (1933) in a communication on the assimilation of nitrogen by yeast, emphasises the importance of the seed yeast for yeast growth in relation to strain, physiological state and quantity. He worked with different brewer's yeasts and his experimental conditions were essentially different from those in the present investigation, without special aeration of the substrate and without additions. For this reason his work will not be further discussed here. BRAUN and PFUNDT (1936), in their paper "The production of baker's yeast by the addition and aeration processes", emphasise the experimental difficulties arising from the nature of the seed yeast. NIELSEN (1935) points out the importance for the yeast growth of the contents of growth substances in the seed yeast and the amount of this yeast used for inoculation. This author worked, as did THORNE, with brewer's yeast in conical flasks, without special aeration and without continuous addition. He showed that if the seed yeast was grown on a substrate rich in growth substances, it became itself rich in these substances. This led to the conclusion that such a yeast can grow at the expense of its own growth substances on a substrate free of such materials. Hence the conditions under which the seed yeast is cultivated play an important part in determining the results of cultivation experiments carried out with this yeast. NIELSEN also demonstrated the importance of the quantity of yeast used for inoculation, showing that the yeast reproduced at a relatively lower rate, the greater the amount of seed yeast employed. A yeast poor in growth substances would not grow better on a growth-substance free substrate by virtue of the fact that a larger quantity was used for inoculation. NILSSON, ENEBO, KARSVIK and LUNDIN (1943), in their paper "Improved yields in compressed yeast manufacture by the addition of straw extract", emphasise that "the nature of the seed yeast may influence to a considerable degree the course of the cultivation and the yield of yeast". For experiments in a given series these authors therefore employed yeast from one and the same batch, in the form of ordinary commercial yeast.

In the present investigation the seed yeast employed was *Saccharomyces cerevisiae* of strain D from the Svenska Jästfabriks AB. This yeast is prepared with molasses and nutrient salts by the continuous process in several consecutive stages. These are termed Z I–Z V. The Z V stage corresponds to the ordinary commercial yeast, while stages Z I–Z IV represent the seed yeast stages for stage Z V. The first and second stages are carried out anaerobically, the third under weak aeration and the fourth and fifth stages under strong aeration. In conformity with the practice of BOREI and SJÖDÉN (1943 a, b) and of BRANDT (1945) the cultivation stages are also denoted by Z I–Z V in the present account. BRANDT gives the characteristics of the different stages, with respect both to their manner of preparation and to their chemical and physiological nature, so that it will be unnecessary to go further into the matter here.

A series of yeast cultivations were carried out in the laboratory with materials from the different stages as seed yeasts, in order to establish which cultivation

Table 18

Cultivation experiments with yeasts from different cultivation stages as seed yeast.

Nutrient medium: saccharose.
Cultivation process: cf. p. 22.
Total sugar used: 10 g. Total volume: 760 ml.

Amount of seed yeast, g dry weight	Yield of yeast, less seed yeast (g dry weight per 100 g saccharose)				
	Z I	Z II	Z III	Z IV	Z V
1.25	35.2	45.2	46.2	17.6	12.2

stage was best suited to serve as seed yeast and how the different stages differed one from the other in their yeast-forming capacities. The results of this experiment are given in Table 18.

The outcome of this experiment, which has repeatedly been confirmed, is of the greatest value in showing the influence of the seed yeast upon yeast cultivations. It is clear from the table that the cultivation stage from which the seed yeast is taken is of paramount importance in determining the yield.

It was now necessary to determine the reason for this great difference between the first three and last two cultivation stages. The primary question was whether the cause lay in the physiological state of the yeast, varied by differences in the enzyme systems, or in substances of non-enzymic character partaking in the metabolism.

After NEUBERG (1927) demonstrated that the decarboxylation of pyruvic acid constituted an intermediate stage in the alcoholic fermentation, numerous investigations were carried out on the turnover of pyruvic acid by yeast. NEUBERG, as well as HÄGGLUND and AHLBOM (1927), worked with dry yeast and yeast juices, showing that these fermented pyruvic acid more rapidly than did living yeast cells. This has been explained by a permeability barrier for pyruvic acid at the cell surface. WIELAND and co-workers (1933, 1939) found in many yeasts, especially brewer's yeast, a respiration of pyruvic acid. RUNNSTRÖM and SPERBER (1941) in their study of the pyruvic acid turnover of yeast, worked with baker's yeast. Two types different from the technical viewpoint were employed, namely a yeast cultivated anaerobically and one prepared under vigorous aeration by the continuous process. The two types showed characteristic differences in their behaviour towards pyruvic acid. For the aerobically cultivated yeast the respiratory quotient was 1.3 and for the anaerobically cultivated organism 1.54. This high RQ value indicated that an oxidation product had been accumulated by the baker's yeast in its turnover of pyruvic acid. It was not possible, however, to demonstrate a simple decarboxylation of the acid.

In a subsequent investigation, RUNNSTRÖM and BRANDT (1941) endeavoured to determine whether such a decarboxylation might constitute an intermediate stage in the degradation of pyruvic acid by baker's yeast. In experiments where, among other things, the respiration was inhibited by carbon monoxide, they actually confirmed this decarboxylation as an intermediate process. They interpreted an increase in RQ to above 2 as a sign of a pure decarboxylation: $CH_3 \cdot CO \cdot COOH \rightarrow CH_3 \cdot CHO + CO_2$, although they were unable to confirm an accumulation of acetaldehyde. In experiments with destroyed baker's yeast, RUNNSTRÖM, BRANDT and MARcuse (1943) made a direct demonstration of the formation of acetaldehyde in the pyruvic acid breakdown, which indicated the presence of a carboxylase in the yeast. A previous experiment by Lynen (1939) with brewer's yeast of a similar type gave similar results. Runnström and Marcuse (1944) further investigated the dependence of the pyruvic acid turnover by baker's yeast upon the physiological state of yeasts cultivated under different conditions. The yeasts principally studied were those cultivated under anaerobic conditions and aerobic conditions of various intensities. They came to the conclusion that the quantitative occurrence of the substances determining the pyruvic acid turnover was dependent upon the "pre-history," i.e. the conditions of cultivation, of the yeast. Borei (1942) studied the endogenous respiration of baker's yeast cultivated under strongly aerobic conditions and found that this consisted of two components, one dependent upon the consumption of substrate, the so-called "monomolecular respiratory component", and the other dependent upon enzymes, the "constant respiratory component". Borei and Lindvall (1943) then showed that the endogenous respiration was affected by pH, the constant component increasing greatly over the pH range 4–6.5, while the monomolecular component remained constant. The RQ was also determined by pH. At pH 5 it was about unity, while at higher pH values it became less than unity. In a study of the cytochrome content of top yeast, Borei and Sjödén (1943) showed that this was also dependent upon the conditions of cultivation. Anaerobically cultivated yeasts showed considerably lower contents of cytochrome c than did those cultivated under strongly aerobic conditions.

All these investigations indicate that the enzyme systems of the yeast are of the highest importance in determining the course of the sugar breakdown. Whether, however, the parallel synthesis of cell substance is determined by the same systems, cannot be ascertained on the basis of our present knowledge.

Yeast cultivation experiments were next made with different quantities of seed yeast from the different cultivation stages in order to determine whether the yeast-forming capacities of the stages could be equalised by increased amounts of seed yeast, and hence of enzymes.

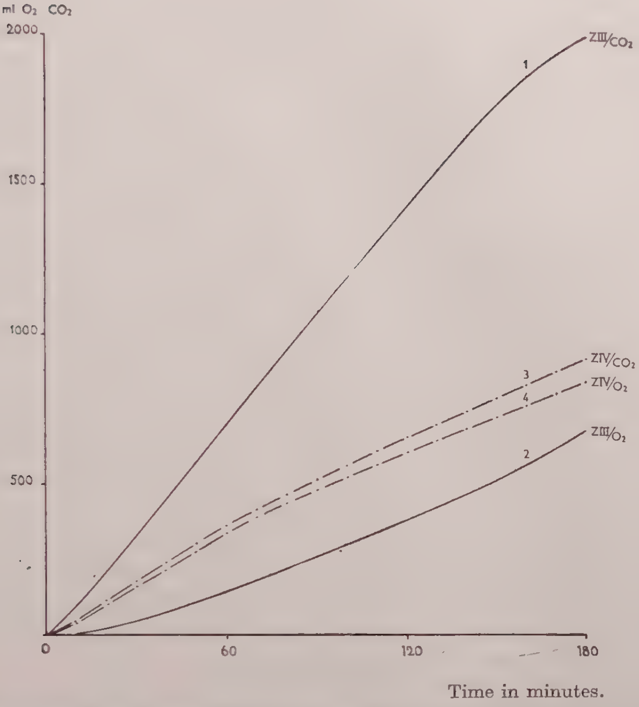
The results of some of these cultivations are set out in Table 19.

Table 19

Cultivation experiments with different amounts of the various cultivation stages as seed yeast.

Nutrient medium: saccharose.
Cultivation process: cf. p. 22.

Weight of 25 % seed yeast, g	Yield of yeast, less seed yeast, g dry weight per 100 g saccharose			
	Z II	Z III	Z IV	Z V
3	32.1	25.4	8.4	6.1
4	45.9	43.0	10.8	9.0
5	45.2	46.2	17.6	12.2
6	45.5	46.3	31.3	17.9
7	45.6	46.0	40.0	28.9
8	45.3	46.1	41.6	35.4
9	44.4	45.9	42.0	40.5
10	44.0	46.3	41.8	40.2



Graph 3. Respiration and fermentation of yeasts from two different cultivation stages, Z III and Z IV.

These results show quite unambiguously that the yeast growth is determined by the quantity of seed yeast employed. It is therefore possible in some degree to compensate for the low proliferating power of a seed yeast by adjusting its quantity.

A further point arising from the table is that a certain maximum yield cannot be exceeded under the given conditions by further increases in the quantity of seed yeast.

In an attempt to establish whether this behaviour was due to different enzyme concentrations in the different yeasts, experiments were performed in which the respiration and fermentation of yeasts of the various stages were studied, and these "enzymic characteristics" compared with the synthetic powers of the respective organisms (cf. also Runnström and Marcuse 1944).

In the present case it was clearly impossible to make a detailed study of the different enzymes, but only the enzyme complexes governing the respiration and fermentation *in toto* could be considered. In this connection reference may be made to the investigations of Borei and Sjödén (1943 a, b), who determined the cytochrome content of different stages and found that this was about four times as high in the aerobically cultivated stages as in the first anaerobic stage. In his studies of the fluoride inhibition of respiration of the different stages, Borei (1945) also showed that the intensity of the respiration varied from type to type.

In the following experiments the oxygen consumption and carbon dioxide formation were determined manometrically by the Warburg procedure. The two parallel manometers described by Warburg and Yabusoe (1924) were employed. In one of these the carbon dioxide was absorbed with 10 % KOH (0.3 ml in the central cup). The pressure variations then indicated the oxygen consumption. Knowing this value, it was possible to calculate the CO_2 formation from the pressure recorded by the second apparatus, in which the carbon dioxide was not absorbed. The total volume of solution was usually 2.4 ml. The pH was generally maintained at 5. The buffer employed (0.083 M succinic acid-succinate) retained no carbon dioxide in chemical combination. In each vessel 0.3 ml of a 3.33 % glucose solution was acted upon with 20 mg yeast, suspended in 1 ml buffer solution.

Graph 3 shows the respiration and fermentation curves for two types of yeast, namely an anaerobically cultivated Z III and an aerobically cultivated Z IV. Curves 1 and 3 show the CO_2 evolution and curves 2 and 4 the oxygen consumption for Z III and Z IV respectively. The differences in fermentation rate and oxygen consumption for the two types are obvious. Curve 1 (Z III) rises steeply almost from the beginning, while curve 3 (Z IV) rises more slowly, without ever approaching the fermentation rate of Z III. It will also be observed that Z III uses less oxygen than does Z IV. This must have a favourable effect upon the increase of cell substance, since we know from the respirometer experiments that lower oxygen consumption results in an increased yield of yeast.

Several experiments of the same type were carried out with the Z III and Z IV yeasts and on each occasion the relative positions of the curves were similar. This indicated considerable reproducibility of the enzyme complex. Experiments were therefore planned to compare the respiratory and fermentation rates of the Z III yeast with its yeast-forming efficiency. The respiratory quotient RQ was selected as a standard of reference, i.e. the ratio of carbon dioxide evolved to oxygen consumed after 90 minutes. The value was calculated after subtraction of the corresponding CO_2 and O_2 values after 30 minutes, since this period could be regarded as induction time, until the passage of which the oxidation velocity was not constant. The question now arose whether a larger RQ, corresponding to an increased evolution of carbon dioxide and a decreased oxygen consumption, was accompanied by an increase in the formation of cell substance, i.e. an increased yield of yeast.

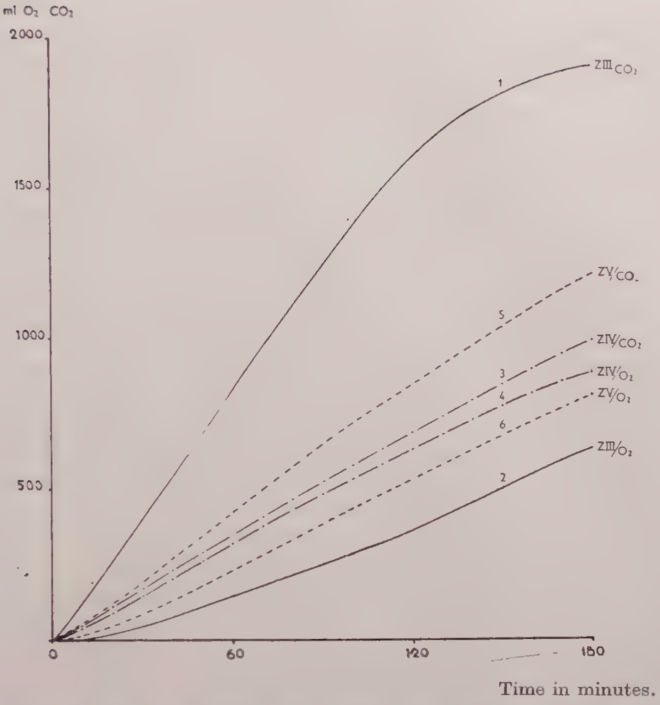
Table 20 shows the results of some yeast cultivations with the same molasses as substrate and with Z III as seed yeast. Warburg determinations were carried out simultaneously with the same seed yeast for the evaluation of the respiratory quotient.

The results show that there is no correlation between the respiratory quotient and the yield of yeast. It is, at the same time, remarkable that RQ varies so widely for different samples of one and the same type of yeast, i.e. between 1.86 and 3.00.

If the respiratory and fermentative properties of yeasts from the different fermentation stages are studied, it appears that these are not exclusively dependent upon the mode of cultivation, aerobic or anaerobic. A continued aerobic cultivation of the Z IV yeast, in which the fifth stage, Z V, is produced, results in a greater anaerobic fermentation power, i.e. a higher evolution of CO_2, but a lower oxygen consumption. It therefore bears more resemblance to a Z III yeast than to the Z IV yeast cultivated under strongly aerobic conditions.

Graph 4 gives a clear representation of the respiratory and fermentative properties of the various types of yeast.

Curve 5, representing the carbon dioxide evolution of the Z V yeast, lies closer to the corresponding curve for Z III (curve 1) than does that for Z IV (curve 3).



Graph 4. Respiration and fermentation of yeasts from three different cultivation stages, Z III, Z IV, Z V.

Table 20

Cultivation experiments with different Z III seed yeasts.

Substrate: molasses.
Cultivation process: cf. p. 22.

RQ	Yeast yield, less seed yeast, g dry weight per 100 g sugar
1.86	49.4
2.29	49.3
2.41	48.7
2.68	48.6
2.83	48.4
2.83	46.0
2.94	48.7
2.98	48.6
3.00	46.7

The same applies to the oxygen consumptions for Z V, Z III and Z IV, represented by curves 6, 2 and 4 respectively.

If the fermentative properties of the yeast determined the rate of growth, it would be expected that the Z V type would give a higher yield than the Z IV. There is, however, no such correlation, as has been established in numerous experiments.

It may therefore be stated with certainty that the enzyme complexes regulating the respiration and fermentation of the yeast are not responsible for the differences in rate of growth of yeasts from the different cultivation stages, and hence cannot be taken as characteristic of this rate.

Similar experiments with beet sugar molasses and seed yeasts from the different stages gave valuable information regarding the rates of growth.

In Table 21 the yields found in these experiments, calculated for 100 g of molasses sugar, are shown for increasing amounts of the different yeasts.

Table 21

Cultivation experiments on beet sugar molasses with increasing quantities of seed yeasts from the different cultivation stages.

Nutrient medium: molasses.
Cultivation process: cf. p. 22.

Weight of 25 % seed yeast, g	Yield of yeast, less seed yeast, g dry weight per 100 g sugar			
	Z II	Z III	Z IV	Z V
3	54.3	54.3	51.1	42.2
4	55.7	55.6	53.3	49.1
5	55.8	54.4	53.7	51.2
6	54.8	53.7	52.6	52.5
7	54.9	54.1	52.9	53.4
8	53.3	53.1	53.4	52.0
9	54.5	53.5	53.5	53.0
10	52.7	51.5	52.7	52.0

The table shows that in this case also there are differences in the growth rates of yeasts from the different stages. This is especially noticeable when the quantities of seed yeast are small, even though the differences here are lower than in the corresponding cultivations on saccharose.

It will also be seen, as in the experiments with saccharose, that a maximum yield is reached which cannot be exceeded by further increases in the amount of seed yeast. However, as far as the yield is concerned, the quantity of seed yeast is not as important in cultivations on beet sugar molasses as in those on saccharose. This is probably due to the fact that the molasses contains substances of importance to yeast growth in such quantities that an increase in the concentration brought about by augmenting the amount of seed yeast is without effect. Substances occurring both in yeast and in beet sugar molasses that may be of importance for the growth of yeast, include amino acids and biological growth factors.

7. The effect of amino acids on yeast growth

Numerous works are to be found in the literature concerning the significance of amino acids in yeast growth. The most important of the older works in this sphere are those of Ehrlich (1906, 1908, 1914, 1927) and Pringsheim (1907, 1908). In the view of these workers, when an amino acid is provided as the sole nitrogen source to growing yeast in a synthetic substrate consisting of sugar, nutrient salts and certain growth factors, it is converted to the corresponding alcohol with loss of carbon dioxide and ammonia. The latter is utilised by the yeast in the synthesis of protein.

Thus, only such amino acids should be of use, as can undergo the Ehrlich deamination. Furthermore, these amino acids should be of equal value as nitrogen sources, since only the resultant ammonia could be turned to account by the growing yeast.

This, however, is not the case. It has been convincingly shown by several workers, including Comrie (1929), Thorne (1933, 1937, 1939, 1941), Nielsen (1936, 1938), Nielsen and Hartelius (1938 a, b, c, 1939) and the present author (unpublished) that there are definite differences in nutritive value among the assimilable amino caids. In a paper entitled "The growth and fermentation of some top fermentation brewery yeasts with amino acids as nutrients", Thorne (1942) classified the amino acids in four groups with clearly different effects. He also found, and this has also been pointed out by Nielsen and Hartelius (1938 b), that the strain of yeast is of importance in determining the nutritive value of amino acids.

The utilisation of nitrogen is characteristic of the nitrogen compounds concerned, provided that these are simultaneously present in equivalent concentrations. Thus, it proved that when glycine, alanine, leucine and ammonium phosphate were present in the substrate, the nitrogen of the amino acids was assimilated more slowly than that of the ammonium salt. Arginine nitrogen was assimilated at a moderate rate, and asparagine and aspartic acid nitrogen more rapidly. If the proportion of amino to ammonium nitrogen is so adjusted that the amino nitrogen is predominant, the percentage of assimilable amino nitrogen also increases. Thorne (1944, 1945) also showed that mixtures of several amino acids had a greater effect upon the yeast growth than had the individual acids. Nielsen (1936, 1938 a, b, c), in several investigations of nitrogen assimilation during the growth of yeast, studied the effects of 27 amino acids. He confirmed the results of Thorne (1933) in the cases examined by the latter. The other amino acids showed various behaviours with regard to their assimilation by yeast. Williams and Rohrman (1936) and Lash Miller (1936) showed that β-alanine acted on yeast as a growth factor. Nielsen and Hartelius (1938 b) confirmed this, provided that beer wort was added as a supplementary source of growth factors. The same authors (1938 c) showed that the growth factor action of β-alanine was associated with the presence of asparagine or aspartic acid and glutamic acid. β-alanine alone is inactive as a growth factor and even has a toxic action in quite low concentrations.

Another study of the value of amino acids as nitrogen sources for yeast was made by Hartelius (1939). This author cultivated yeast in the presence of ammonium sulphate and an amino acid, as distinct from Nielsen, who always used the amino acid under investigation as the sole nitrogen source. During its growth the yeast was hence free to choose between ammonia and amino nitrogen. Essential differences in the assimilability of the amino acids continued to appear, whether these were added together with ammonia nitrogen or alone to the growing yeast. Nielsen and Hartelius (1939) later showed that the action of β-alanine as a growth factor was not only conditional on the presence of asparagine, aspartic acid and glutamic acid, but also on that of aneurin. In 1940 Nielsen and Dagys demonstrated that asparagine and glutamic acid could be replaced by citric acid, succinic acid, tartaric acid or malic acid in conjunction with β-alanine as growth factor. The sensitivity of the yeast towards β-alanine proved to be independent of which accompanying material was employed. In a work on the mode of action of β-alanine as a growth factor, Nielsen (1940) expresses the opinion that this compound forms part of a protein complex. In a paper on the action of amino acids as growth factors, published in 1941, Nielsen comes to the conclusion that of 31 amino acids and related compounds investigated, only seven can be said to have any effect upon the growth of yeast. These are β-alanine, lysine, arginine, methionine, aspartic acid, asparagine and glutamic acid. The concentrations in which the various amino acids act are quite different, so that in many cases, when higher concentrations are necessary, it is no longer possible to describe the acids concerned as growth factors, but rather as nutrients with specific effects, which may be employed in the synthesis of certain special proteins.

All these investigations clearly indicate that the amino acids play an important part in the synthesis of yeast protein. ROINE (1946) showed that the greater part of the acid-soluble nitrogen of a yeast consisted of amino acid nitrogen. It appeared of interest to determine whether the yeasts from the different cultivation stages differed in their contents of acid-soluble nitrogen. The determinations were made on a number of yeasts of the different types by the procedure described by ROINE (1947). The mean values of ten determinations on each yeast gave the following acid-soluble nitrogen contents per 100 g 25 % yeast: for Z II, 0.36 g, Z III 0.36 g, Z IV 0.30 g and Z V 0.30 g. The values for the two earlier stages were therefore somewhat higher than those for the later. It must, however, be borne in mind that the total nitrogen content is also greater in Z II and Z III. This was 2.41 g per 100 g 25 % yeast for the samples in question, while the values for Z IV and Z V were 2.01 and 1.78 g respectively. The percentage content of acid-soluble nitrogen thus decreases with yeast cultivated through an increasing number of stages. The table on p. 41 shows, however, that the rate of growth does not increase correspondingly. It will be remembered from the experiments with increasing quantities of seed yeast from the different stages, described on pp. 42 and 46, that the rates of growth can be greatly influenced by increased quantities of seed yeast. This may possibly be due to increased addition of acid-soluble nitrogen, i.e. of amino acids. The experimental material is still too small to determine whether or not this is the case, so that only the possibility will be indicated here.

Chapter III. Growth factors and their importance in the growth of yeast

1. Review of previous literature

The importance and necessity of organic growth substances in the synthesis of yeast have constituted an object of investigation since the time of LIEBIG and PASTEUR. In 1860 PASTEUR, in his work "Mémoire sur la fermentation alcoolique", maintained that for the growth of yeast only its ash, fermentable sugar and inorganic nitrogen were necessary. LIEBIG (1871) opposed this theory, saying that if PASTEUR's directions were precisely followed, the yeast would be able neither to ferment nor to proliferate.

The theory of PASTEUR, however, stood its ground for at least thirty years. WILDIERS (1901) met with strong opposition when he revived LIEBIG's objection and stated that apart from sugar, ash and inorganic nitrogen, one or several specific organic substances, which he termed "bios", were essential for growth. He found this growth substance in both beer wort and yeast. It was insoluble in water and dilute alcohol and ether, not precipitable by lead acetate, but adsorbable on carbon and fullers earth.

FERNBACH (1902) and WINDISCH (1902) concluded that WILDIERS' substrate must have contained traces of toxic metal salts. PRINGSHEIM (1906) believed that the yeast must first acclimatise itself to its substrate. He admitted, however, that ordinary commercial sugar, as distinct from pure saccharose, contained traces of organic substances that stimulated fermentation and growth. AMAND (1902, 1904) was able to disprove the toxicity theory of FERNBACH and WINDISCH experimentally, and showed that bios was consumed during the growth of yeast.

DEVLOO (1906) studied the occurrence of bios in natural raw materials. IDE (1907) refuted PRINGSHEIM's acclimatisation hypothesis, showing that yeast had not acclimatised itself, even after long cultivation in a bios-free substrate.

KOSSOWICZ (1903, 1906) found that yeast developed normally in a synthetic medium in the presence of bacteria or moulds. He supported WILDIERS' statement that certain organic substances, even in traces, exerted a strong influence on the proliferating power of yeast. FUNK and FREEDMAN (1923) showed that ordinary cane sugar contained an organic growth factor which could be removed by recrystallising the sugar from alcohol three times.

COPPING (1929) showed that the different strains of *Saccharomyces cerevisiae* could differ greatly in proliferation power.

WILLIAMS and co-workers (1927, 1929, 1931) confirmed the findings of COPPING. In 1923 and subsequent years a number of publications appeared, including those of EDDY, KERR and WILLIAMS (1924) and SUZUKI and HAMAMURA (1928), which all indicated that bios was not a single substance. FULMER and co-workers (1924) also recognised the multiple nature of bios. LUCAS (1924), by treatment of bios with alcoholic barium hydroxide or with basic lead acetate, was able to separate it into two components, each of low activity when employed separately. The factor found in the precipitate he named bios I, and that in the filtrate bios II. In 1928 EASTCOTT isolated bios I from tea dust and it proved to be identical with inositol. This was the first report of the chemical identity of a growth factor isolated from natural material. A start was now made at attacking the bios problem preparatively. By adsorption from vegetable or animal extracts on fullers earth, WILLIAMS, WARNER and ROEHM (1929) separated bios into several components. Further they were able to show that the factor adsorbed on fullers earth could be replaced by the antineuritic vitamin first isolated by JANSEN and DONATH (1926), aneurine. Definite effects on growth were perceptible even in a dilution of 1 part of aneurine to 100 million parts of substrate. Later, SCHULTZ, ATKIN and FREY (1928) showed that aneurine was a growth factor for many strains of yeast, but had a growth-inhibitory effect on others. WILLIAMS and TREUESDAIL (1931) then succeeded in resolving, by electrodialysis, the bios essential to WILDIERS' yeast into two mutually complementary factors. In 1932 KÖGL commenced to make a detailed study of bios II, the component remaining in the filtrate after adsorption of bios on fullers earth. This research resulted in the isolation and chemical identification of a new crystalline growth factor, which KÖGL and TÖNNIS (1936) named biotin. This substance has an extremely powerful effect upon the cell proliferation of yeast. Even in a dilution of $1:4\times10^{11}$ KÖGL was able to demonstrate a decided effect upon the yeast growth.

For maximum growth, however, yeast requires several other bios factors in addition to biotin. This KÖGL showed in experiments on pure synthetic substrate with a yeast especially poor in bios, a distillery top yeast of "strain M" from the Institut für Gärungsgewerbe in Berlin. An overdosage of biotin under definite experimental conditions gave a growth of only 250–300 % in 5 hours, while after addition of boiled yeast juice a maximum growth of about 600 % was obtained in the same period.

Williams and his co-workers (1933) reported a growth factor of general biological occurrence which greatly stimulated the growth of yeast. This they named pantothenic acid. Williams and Major (1940) determined the structure of this substance and effected its synthesis. Schultz, Atkin and Frey (1939) and, independently, Eakin and Williams (1939) studied the growth effect of pyridoxine on *Saccharomyces cerevisiae.*

In an investigation with three different strains of *Saccharomyces cerevisiae*, Williams, Eakin and Snell (1940) studied the growth effects of inositol, aneurine, biotin, pantothenic acid and pyridoxine. Large differences were found in the requirements of the different strains for growth factors. Two grew without inositol, while aneurine was required by only one strain. Biotin alone had only a weak action, but in suitable combination its stimulating effect was extraordinarily strong even at very low concentrations. Pantothenic acid was essential to all the strains investigated, while pyridoxine was relatively unimportant.

Of the many growth factors of importance to the growth of microorganisms, five have proved to be of special significance for the growth of yeast, namely biotin, pantothenic acid, aneurine, inositol and pyridoxine.

Several reports have appeared in the literature which are directly concerned with the vitamin requirements of numerous strains of yeast. Burkholder (1943) described experiments with 38 different strains, of which 15 required aneurine, 14 pantothenic acid, 36 biotin, 6 nicotinic acid, 4 inositol and 6 pyridoxine. In a similar study of 33 strains of yeast and 17 strains of moulds, Burkholder and Moyer (1943) showed that most of these required biotin and aneurine. Burkholder, McVeigh and Moyer (1944) reported on the vitamin requirements of a further 163 strains of yeast, of which 78 needed biotin, 33 aneurine, 30 pantothenic acid, 15 inositol, 13 nicotinic acid and 13 pyridoxine. Leonian and Lilly (1942) studied the needs of ten strains of *Saccharomyces cerevisiae.* Only one proved to be indifferent to aneurine and four indifferent to inositol. All showed a weakening in growth when pantothenic acid was lacking and no growth occurred when biotin was omitted. No strain was sensitive to the omission of pyridoxine. When, however, pyridoxine and aneurine, or aneurine and inositol were omitted, the growth of some strains was weakened. Similar investigations have been made by Rogosa (1944) and by Emery, McLeod and Robinson (1946).

Illies (1937, 1938, 1939) studied the occurrence of growth factors in beet sugar molasses and cane sugar molasses, as well as in other raw materials employed in the fermentation industry, such as malt sprout extract. From comparative experiments with pure inorganic nutrition, inorganic and synthetic organic nutrition and with pure molasses solution and inorganic nitrogen, he showed that molasses contained various materials with the character of growth factors. The cane molasses proved to be superior to beet molasses in this respect. Wendel (1941) writes on the practical importance of growth factors in the production of baker's yeast from molasses, with special reference to the changes in the growth factor contents of the substrate and of the yeast during the manufacture. From Nielsen's school at the Carlsberg Institute in Copenhagen a large number of communications have been made concerning the growth-promoting properties of amino acids towards yeast. It should be borne in mind, as Nielsen and Hartelius (1938 a) have pointed out, that a single amino acid may show no growth-promoting effect, or even toxic effects, whereas it may exert a marked growth-promoting action in combination with other amino acids or complementary substances.

WILLIAMS and ROHRMAN (1936) and LASH MILLER (1936) regard β-alanine in itself as a growth factor for yeast, while NIELSEN and DAGYS (1940) showed that for β-alanine to show an effect on yeast, other substances must be added, namely, apart from biotin and aneurine, also asparagine and glutamic acid. These latter compounds may be replaced by citric, succinic, tartaric or malic acid, when the sensitivity of yeast towards β-alanine becomes even greater. WAGNER (1936) also studied the effects of amino acids, alone and in combination with other substances influencing the growth of yeast.

2. Aerobic laboratory yeast cultivations with addition of various growth factors

a) Addition of biotin

All the above-described investigations, largely carried out under anaerobic conditions, clearly showed the importance of growth factors for yeast growth. It was therefore of great interest to study the influence of individual substances, known to have growth-promoting properties, on yeast growth under strongly aerobic conditions. In order to exclude the growth factor content of the nutrient medium, as for example in molasses, the experiments were first carried out with synthetic substrates in the manner described for the standard experiment on pp. 22 and 23.

The synthetic substrate had the following composition: 34 g saccharose, 5.4 g K_2SO_4, 1.0 g NaCl, 0.1 g $MgSO_4$, 0.2 g $CaCl_2$, all dissolved in 1000 ml of ordinary tap water. During the cultivation the following nutrient salts were added: 1.1 g ammonium sulphate, 0.8 g diammonium phosphate, together with 0.8 ml 25 % ammonia, all per 1000 ml substrate.

The growth factors investigated were biotin, pantothenic acid, aneurine, pyridoxine and inositol. Previous cultivation experiments on synthetic substrate had shown the importance of the amount of seed yeast for the growth rate (cf. p. 42). For the first experiment on the effect of biotin on yeast growth under strongly aerobic conditions, the seed yeast employed was therefore Z IV, of 25 % dry weight, in quantities of 5, 6 and 7 g. Table 19 on p. 42 shows clearly that with these quantities there is a definite deficit of yield-determining factors. It was hence attempted to compensate for this deficit by the addition of biotin.

The results are given in Table 22.

The following conclusions may be drawn from these results:

1. The yield of yeast is dependent upon the amount of seed yeast (cf. p. 42).
2. Addition of biotin considerably increases the yield.
3. This effect of biotin is no longer increased above a certain concentration of the growth factor.
4. The increase in yield obtained by increasing the amount of seed yeast may also be achieved by addition of biotin.

This experiment shows quite convincingly that the growth of *Saccharomyces cerevisiae* is greatly influenced by biotin, also under strongly aerobic conditions.

In the next experiments, in order to determine the necessary concentration of biotin, the amount was varied over the range 0.1 μg–200 μg per 10 g sugar. The procedure was otherwise the same as before.

Table 22

Yeast cultivation experiment in synthetic substrate with addition of biotin.

Cultivation process: cf. p. 22.

Amount of 25 % seed yeast, g	Biotin μg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	18.4
6	0	26.8
7	0	39.2
5	200	45.2
6	200	46.2
7	200	45.7
5	400	46.2
5	600	45.5

Table 23

Yeast cultivation experiment on synthetic substrate with addition of biotin.

Cultivation process: cf. p. 22.

Amount of 25 % seed yeast, g	Biotin μg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	16.1
5	0.1	20.9
5	0.2	28.4
5	0.4	35.5
5	0.6	43.1
5	0.8	44.3
5	1.0	44.9
5	2.0	46.2
5	10.0	46.8
5	20.0	47.1
5	50.0	46.3
5	100.0	46.7
5	200.0	47.3

The importance of biotin for yeast growth is obvious from the results in Table 23. The growth-promoting effect is clear even at concentrations of biotin as low as 0.1 μg per 10 g sugar. The total volume of the cultivation is 750 ml, which corresponds to a biotin dilution of $1 : 7.5 \cdot 10^9$. Tables 22 and 23 show that under the experimental conditions employed, with Z IV as seed yeast and with addition of biotin, a maximum yield of 46–47 g dry yeast substance can be obtained from 100 g sugar. This is about 10 % higher than the yield obtained with the same seed yeast without addition of biotin (p. 42). However, the yield represents that attainable with Z III as seed yeast without addition of biotin. In experiments with different seed yeasts, Z III has proved to be the most valuable with respect to yield.

These results, which were repeatedly confirmed, were of the highest importance, in that they provided a direct indication of the growth factor most important for yeast growth under the given experimental conditions, namely biotin. It was subsequently shown that other growth factors such as pyridoxine, aneurine, pantothenic acid and inositol were of much less importance for the growth of the yeast employed here.

It was now important to determine whether yeasts from the different stages Z II - Z V which had been used in the cultivation experiments on synthetic substrates (p. 42), differed in their biotin contents and whether the different growth powers could be attributed to such a variation.

b) Determination of biotin in *Saccharomyces cerevisiae*

Microbiological growth methods are especially suited to the quantitative estimation of biotin. Kögl and Tönnis (1936) worked out such a method, employing a top yeast of strain M as their test organism. The growth was determined nephelometrically after a five-hour incubation period.

Snell, Eakin and Williams (1940) employed for the estimation of biotin a strain of *Saccharomyces cerevisiae* isolated from Fleischmann baker's yeast, and determined the growth with a nephelometer after 16 hours. Nielsen and Hartelius (1942) described a method in which compressed yeast was used as test organism.

Lampen, Kline and Peterson (1941) used *Chlostridium butyricum* as test organism. West and Woglom (1941, 1942) worked with *Rhizobium trifolii* or *Bact. radicicola*, and Lindeberg (1946) with *Collybia dryophila*. Ryan, Beadle and Tatum (1943), in experiments on the influence of various amounts of biotin on the growth rate of *Neurospora*, obtained excellent results by the "tube method". *Neurospora* grows on a purely synthetic substrate which, apart from inorganic salts, an inorganic source of nitrogen and sugar, contains only biotin. With the exception of biotin, *Neurospora* synthesises all necessary vitamins, amino acids and other essential factors. Melin and Norkrans (1948) describe other methods for the estimation of biotin.

In the present investigation the biotin estimations were carried out with a strain of *Neurospora*, namely *Neurospora crassa* No. 37401.[1]

Preparation of the conidial suspension of *Neurospora crassa*

From a four-day old culture of *Neurospora*, which had been cultivated in tubes on malt agar at 25° C., conidia were transferred by means of a platinum wire to a 300 ml conical flask containing about 250 ml of sterile distilled water. After vigorous shaking the suspension was filtered through a sterile cotton-wool filter in order to remove any mycelia. So that a suspension as free as possible from biotin might be obtained, the conidia were washed by centrifuging the suspension in two sterile centrifuge tubes fitted with rubber stoppers for one hour in an angle centrifuge at about 3500 r. p. m. The sedimented conidia were transferred, with as little liquid as possible, by means of a pipette into a flask containing sterile water. The number of conidia per ml suspension was determined in a haematocytometer, and the suspension was diluted with sterile water until each ml contained about 5×10^4 conidia. The flasks were inoculated with 1 ml of this suspension.

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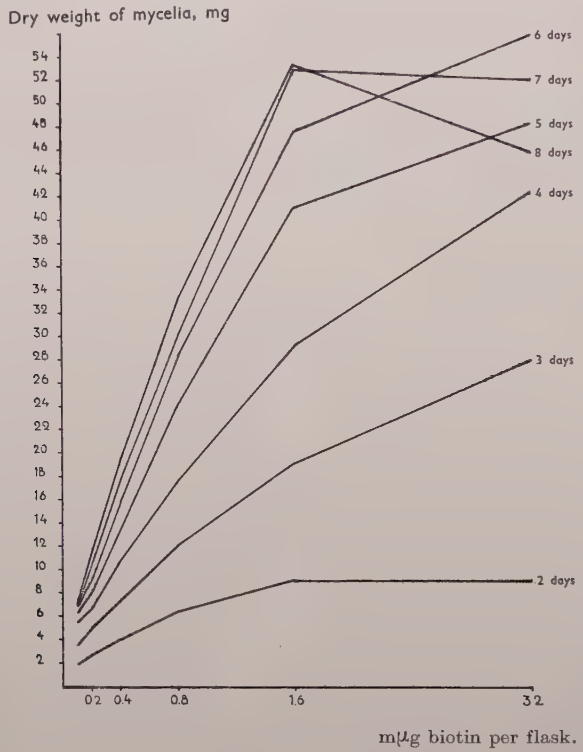
[1] This strain was generously placed at my disposal by Professor Elias Melin, Head of the Institute of Physiological Botany in Upsala. The biotin determinations were carried out under his direction, at the same Institute, by Mrs. Birgitta Norkrans, to whom I should like to take this opportunity of expressing my heartiest thanks.



Graph 5. Growth of *Neurospora crassa* at different biotin concentrations.

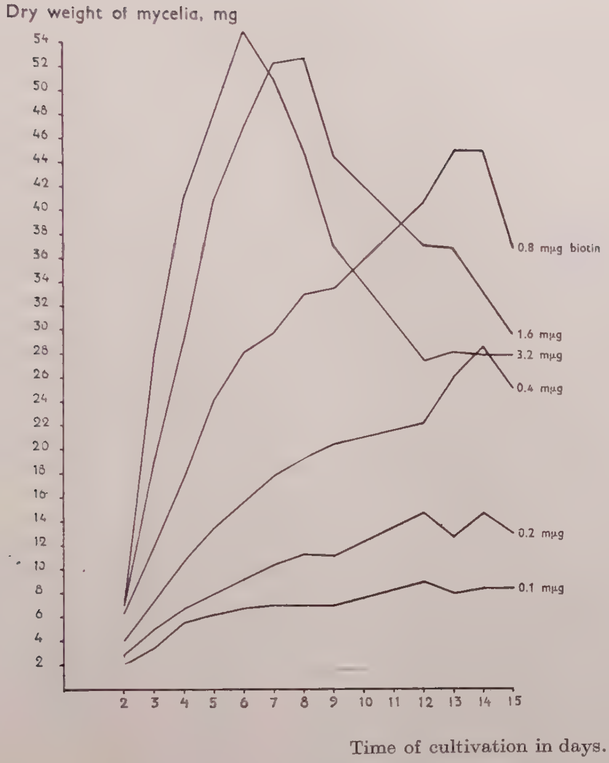
Growth curves for various biotin concentrations were constructed by weighing the mycelia produced after different times in suspensions of known biotin content. From these curves it was then possible to determine the biotin content of an unknown substrate. The strain of *Neurospora* employed was especially suited for biotin assays, since it definitely required biotin for its growth, which proceeded rectilinearly over considerable ranges of time and biotin concentration.

The following procedure was employed in the assays:

Neurospora was cultivated in 100 ml Jena conical flasks on 20 ml of substrate after inoculation with 1 ml of spore suspension containing about 5×10^4 spores per ml. The substrate was complemented according to FRIES (1938) and prepared according to RYAN and co-workers (1943). It had the following composition: 5 g ammonium tartrate, 1 g ammonium nitrate, 1 g primary potassium phosphate, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2$, 0.01 mg B, 0.1 mg Cu, 0.2 mg Fe, 0.02 mg Mn, 0.02 mg Mo, 2.0 mg Zn and 10.0 g glucose per litre. When the *Neurospora* strain 37401 ("inositolless") was employed, the substrate was complemented with 12.5 mg inositol per litre.

The washed mycelia were drained on a fine-meshed metal sieve, washed with distilled water, pressed between filter paper, pre-dried overnight at 50° C. and finally dried to constant weight in weighing bottles at 105° C.

Graphs 5 and 6 show the growth curves of *Neurospora* at different biotin concentrations under the conditions described above.



Graph 6. Growth of *Neurospora crassa* at different biotin concentrations.

The graphs show that the growth curves were most rectilinear for the biotin concentrations 0.2–0.8 mμg per flask and for cultivation periods of 4, 5 and 6 days. At higher biotin concentrations and the same cultivation periods the curves deviated from a rectilinear course. For subsequent biotin assays, therefore, these times and biotin concentrations were chosen. When the biotin content was completely unknown, preliminary experiments were carried out with various dilutions to give an approximate value.

Extraction of biotin from yeast

The first determinations of biotin in various organic materials were made by KÖGL, who extracted the materials with hot water and estimated the biotin in the extracts. Later, other methods of treatment were employed, such as treatment with enzymes or acids and autolysis, GYÖRGYI (1939), GYÖRGYI, KUHN and LEDERER (1939) and SNELL, EAKIN and WILLIAMS (1940).

THOMPSON, EAKIN and WILLIAMS (1941) described comparative extraction experiments on different materials. They showed that, under their experimental conditions, acid hydrolysis generally gave the highest yield of biotin.

c) Biotin contents of yeasts from various cultivation stages and their effects on yeast growth

In the present investigation our first task was to determine whether the biotin contents of the four seed yeasts from different cultivation stages were different. For this purpose a yeast extract was prepared in the following manner:

1 g (wet weight) of yeast was suspended in 100 ml water and autoclaved for 10 minutes at 2 atmospheres. In order to facilitate the subsequent filtration, the suspension was centrifuged. It was then passed through a G 4 Jena glass filter. The yeast residue was again suspended in water, centrifuged and filtered. The combined filtrates were diluted, so that in the highest concentration employed 1 ml corresponded to 0.22 mg dry yeast substance. For the further biotin assays dilutions corresponding to 0.11 and 0.05 mg dry yeast substance per ml were prepared. For the yeast Z V these dilutions proved too large, and concentrations corresponding to 5 and 2.5 mg per ml were used.

The biotin values in Table 24 represent the means from three cultivation periods, 4, 5 and 6 days, and from 2 or 3 concentrations.

Table 24

Biotin contents of yeasts from different cultivation stages.

Cultivation stage	µg biotin per g dry yeast substance
Z II	0.053
Z III	0.055
Z IV	0.017
Z V	0.0026

The table shows conclusively that the yeasts from the various stages differ essentially in biotin content. In view of these results, of the effects of biotin additions in cultivations on synthetic substrates (p. 52) and of the results of the yeast cultivations on synthetic substrates with varying quantities of seed yeasts from the different stages (p. 42), it would seem reasonable to conclude that biotin is one, perhaps the most essential, of the growth factors determining the yeast-forming capacity of a given cultivation stage. A further, and perhaps decisive piece of evidence in favour of this conclusion is provided in the results of the following yeast cultivations with synthetic substrate and seed yeasts from the different stages, which were carried out in two simultaneous groups, with and without addition of biotin (Table 25).

The results in the table show that it is possible to influence greatly, by the addition of biotin to cultivations, the yeast-forming power of the different types of seed yeast. These additions, can indeed, provide very uniform conditions of cultivation for the different yeasts. It would seem that the experimental results explain the uneven yeast-forming capacity of yeasts from the various cultivation stages, on the basis of their different biotin contents.

Table 25

Yeast cultivation experiments with different seed yeasts (cultivation stages Z III–Z V) with and without addition of biotin.

Amount of biotin added: 1 µg per 10 g saccharose.
Cultivation process: cf. p. 21.

Amount of seed yeast, g dry weight	Yeast, yield, less seed yeast, g dry weight per 100 g saccharose					
	Z III		Z IV		Z V	
	without biotin	with biotin	without biotin	with biotin	without biotin	with biotin
1.25	45.3	46.6	22.9	45.9	11.9	45.6

d) Addition of other growth factors

From the literature referred to in the present chapter concerning the part of growth factors in the growth of yeast, it would appear that, apart from biotin, other vitamins such as pyridoxine, aneurine, pantothenic acid and inositol are of importance. An attempt was therefore made to determine whether one or more of these substances were also essential under aerobic conditions for the strain of *Saccharomyces cerevisiae* under investigation.

The experiments were carried out in the same manner as the corresponding experiments with biotin, Z IV being used as seed yeast and cultivated on synthetic substrate.

Tables 26–29 show that under the conditions prevailing in these experiments, only additions of aneurine and inositol affect the yeast yield, while additions of pyridoxine and pantothenic acid are without effect. This may be due to the fact that the seed yeast used had sufficiently high contents of pyridoxine and pantothenic

Table 26

Yeast cultivation experiment on synthetic substrate with additions of pyridoxine.

Cultivation process: cf. p. 21.
Seed yeast: Z IV with 25 % dry weight.

Amount of 25 % seed yeast, g	Pyridoxine, µg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	13.6
5	1	12.8
5	5	13.3
5	10	13.0
5	25	13.3
5	50	13.1
5	100	12.9
5	200	13.0

Table 27

Yeast cultivation experiment on synthetic substrate with additions of aneurine.

Cultivation process: cf. p. 21.
Seed yeast: 5 g Z IV with 25 % dry weight.

Amount of 25 % seed yeast, g	Aneurine, µg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	12.7
5	5	13.1
5	25	13.5
5	50	14.0
5	100	14.5
5	200	15.0
5	400	15.9
5	800	15.6

Table 28

Yeast cultivation experiment on synthetic substrate with additions of pantothenic acid.

Cultivation process: cf. p. 21.
Seed yeast: 5 g Z IV with 25 % dry weight.

Amount of 25 % seed yeast, g	Pantothenic, acid, µg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	13.0
5	10	12.4
5	50	12.7
5	100	12.4
5	250	13.1
5	500	13.1
5	1000	13.3
5	2000	13.1

Table 29

Yeast cultivation experiment on synthetic substrate with additions of inositol.

Cultivation process: cf. p. 21.
Seed yeast: 5 g Z IV with 25 % dry weight.

Amount of 25 % seed yeast, g	Inositol, µg per 10 g sugar	Yeast yield, less seed yeast, g dry weight per 100 g sugar
5	0	13.4
5	10	14.4
5	50	14.2
5	100	14.8
5	200	15.1
5	400	15.1
5	600	15.8
5	800	15.0

acid, so that extra additions were without effect, while the contents of aneurine and inositol were inadequate. A second possibility would be that the strain of *Saccharomyces cerevisiae* in question required no pyridoxine or pantothenic acid for its growth.

Schultz and Atkin (1947) used the bios requirements of various strains of yeast as a standard of characterisation and thus provided a valuable complement to the morphological and physiological methods of classification. Six well-defined growth factors were studied in this connection: inositol, pantothenic acid, biotin, aneurine, pyridoxine and nicotinic acid. For the indication of the requirements of a given yeast, a simple system was proposed in the form of a "bios number". The growth factors were numbered as follows:

1. Inositol.
2. Calcium pantothenate.
3. Biotin.
4. Aneurine.
5. Pyridoxine.
6. Aneurine and pyridoxine.
7. Nicotinic acid.

A yeast with the index number 134 would therefore need for its cultivation under accurately defined conditions inositol, biotin and aneurine. A baker's yeast investigated by Schultz and Atkin from the National Collection of Type Cultures, London, received the number 234. Another baker's yeast from the Fleischmann Laboratories Collection was numbered 1234.

In Table 21 on p. 46, the yields are recorded from cultivation experiments with molasses as substrate and increasing quantities of seed yeast from the different cultivation stages. The different yeast-forming powers of the four seed yeasts are here shown. The cultivations with synthetic substrate and seed yeasts from the four stages, the cultivations with additions of biotin and the biotin assays on the seed yeasts show that the yeast-forming capacities of the different yeasts are determined by their biotin contents. It was therefore of interest to determine whether the molasses employed in the experiments contained biotin in such quantities as would explain, at least in part, the smaller alterations in yeast-forming capacity observed in the cultivations with increasing quantities of the different cultivation stages as seed yeasts.

e) Determination and significance of biotin in molasses

The determination of biotin in molasses was carried out by the microbiological growth method with *Neurospora crassa* in the following manner:

The biotin was extracted by heating the molasses in aqueous solution. 5 g of material (calculated as dry weight) was heated with 250 ml distilled water in a 2 l graduated flask for 10 minutes at 2 kg pressure. After dilution to the mark, this extract was used in several further dilutions in which the basic synthetic substrate was used as diluent. Of the solutions so prepared, quantities of 20 or 30 ml were placed in conical flasks of 100 or 125 ml respectively. After sterilisation for 10 minutes at 2 kg pressure, these were inoculated with 1 ml of spore suspension (cf. p. 53) and kept in an air thermostat at 25° C. Three or four flasks were used for each concentration. After 4, 5 and 6 days the separated mycelia were filtered, washed,

Table 30

Biotin contents of various molasses.

Description of molasses	μg biotin per 100 g molasses	μg biotin per 100 g dry weight of molasses
Swedish raw beet sugar molasses:		
Staffanstorp 1946—47	4.3	5.5
Arlöv-Nässjö » »	3.2	4.2
Hököpinge » »	4.5	5.4
Arlöv » »	3.2	4.2
Mörbylånga » »	3.6	4.7
Roma » »	4.0	5.1
English molasses	4.6	6.1
Cuban sugar cane molasses	10.8	12.9

pressed between filter paper and, after pre-drying at 50°, dried to constant weight at 105° C. Simultaneous series of experiments were performed with 0.1, 0.2, 0.4 and 0.8 mμg biotin per flask. From the results of the dry weight determinations, growth curves were drawn which served for the determination of biotin in the unknown samples.

In Table 30 are shown the biotin contents of some Swedish raw beet sugar molasses, an English raw beet sugar molasses and a cane sugar molasses.

It will be seen from the table that considerable differences may be found among the biotin contents of molasses. It is especially worthy of note that cane sugar molasses contains more than double as much biotin as beet sugar molasses.

Table 31 gives a summary of the yields obtained in yeast cultivation experiments with four different molasses, with and without addition of extra biotin. The amount of the biotin addition was so regulated that more than enough biotin (10 μg), independently of the content of the molasses, was available in each experiment for

Table 31

Yeast cultivation experiments with various molasses, with and without addition of biotin.

Amount of molasses: corresponding to 10 g polarising sugar.
Seed yeast: 5 g Z V with 25 % dry weight.
Biotin addition: 10 μg per 10 g sugar.
Substrate concentration: 1 : 40.
Cultivation process: cf. p. 21.

Description of molasses from experimental record	Yeast yield, less seed yeast, g dry weight per 100 g sugar	
	without added biotin	with added biotin
JO 212 A	44.6	49.8
» » St	42.6	50.4
» » Hö	45.9	49.3
» » A-N	42.3	49.1

the sugar present. The necessary data were taken from Table 23. As seed yeast, a material poor in biotin (Z V) was employed. In each cultivation an amount of molasses corresponding to 10 g sugar was used.

The results in the table show clearly that differences in the molasses may be greatly compensated, with respect to yeast-forming capacity, by addition of extra biotin. Without this addition the difference between the best and the worst molasses is 3.6 %, which is reduced by extra biotin to 1.3 %. This supports the conclusion that the yeast growth is dependent upon the biotin content of the molasses, which may largely compensate for variations in the biotin content of the seed yeast.

As reported at the Biochemical Congress in Cambridge 1949, E. R. Dawson and J. R. Harrrison have also been working with the biotin problem. They have been able to confirm my results on the paramount importance of the biotin for the yeast growth.

f) The influence of natural extracts on yeast growth

When grain worts were replaced by molasses worts in the compressed yeast industry, it was soon found that the latter could be supplemented with advantage not only with phosphates and inorganic nitrogen, but also with expressed juices from various vegetables, or extracts of these, in order to increase the yield of yeast. Use has thus been made of beet or carrot juice, extracts from rice husks and plant sprouts such as malt sprouts, or yeast extracts as additional mash material. Nilsson, Enebo, Karsvik and Lundin (1943) report that improved yields of yeast may be obtained in compressed yeast manufacture by the addition of straw extract or straw hydrolysate.

Several experiments in which potato juice was employed as an additional mash material will be reported. The juice obtained by mincing the potato and pressing out the resultant mash in a hand press was first freed from coagulable protein by boiling or by acidification with sulphuric acid. The completely clear juice thus ob-

Table 32

Content of alcohol-fermentable sugar in potato juices.

Variety of potato	Percentage sugar content of juice, determined		
	after inversion with HCl	after inversion with enzyme	by fermentation
Oval yellow	0.87	0.71	0.69
Ackersegen	1.04	0.91	0.90
Majestic	1.24	1.14	1.12
King George V	0.70	0.53	0.53
Dukker	0.80	0.69	0.69
Early Puritan	0.81	0.64	0.64
Commercial potatoes without indication of variety	—	1.28	—
	—	1.40	—
	—	1.05	—
	—	0.94	—

tained had a dry weight of about 4%. About 3.5% of the dry material was nitrogen, whereof about 70 % was amino nitrogen. The content of sugar fermentable to alcohol, determined by the yeast desaccharification method by MENZINSKY (1943) after inversion with enzyme (cf. Appendix, p. 88), varied rather widely between 0.5 and 1.4 %. In Table 32 are collected some data concerning the sugar contents of potato juices. These were estimated after inversion with hydrochloric acid, after enzymic inversion and by fermentation.

The table shows, among other things, that in the determination of alcohol-fermentable sugar in potato juice the hydrolysis should not be carried out with acid but with enzyme, since the acid also inverts polysaccharides that are not attacked in the fermentation with yeast.

Yeast cultivation experiments

The cultivation experiments were carried out at 27–28° C. Each flask contained a quantity of molasses corresponding to 10 g sugar, ammonium sulphate, diammonium phosphate and ammonia as extra nutrients, 5 g of Z III yeast of 25 % dry weight and various quantities of potato juice. The juice was mixed with the diluted molasses (the stock wort) and the mixture was added in graduated portions during the cultivation.

The sugar content of the potato juice was taken into account in the parallel cultivation without juice by addition of the corresponding amount of pure saccharose. The time of fermentation was 17 hours.

Table 33 summarises the results of some yeast cultivations with raw beet sugar molasses and refined molasses with and without addition of potato juice. The table also gives the protein content of the yeast crops.

The results make it clear that a considerable increase in yield may be achieved in yeast cultivations with molasses as substrate by the addition of potato juice.

Table 33

Yeast cultivation experiments with molasses and addition of potato juice.

Beet sugar molasses: polarisation 44.4 %.
Refined molasses: polarisation 48.4 %.
Nutrient salts: ammonium sulphate, diammonium phosphate, ammonia.
Potato juice: 20 ml per cultivation.
Seed yeast: 5 g Z III with 25 % dry weight.
Wort concentration: 1 : 37.
Cultivation method: cf. p. 22.

Description of molasses	Yeast yield, g dry substance calculated on 100 g polarisable molasses sugar		Protein content of yeast crop as percentage of dry weight	
	without juice	with juice	without juice	with juice
Raw beet sugar	54.7	56.5	46.4	48.4
» » »	54.5	57.9	46.8	47.8
» » »	52.5	56.2	47.4	47.7
» » »	51.9	55.2	47.1	47.4
Refined	51.4	54.1	44.9	46.4
»	50.6	53.3	42.9	46.4
»	49.6	52.0	46.4	46.2
»	49.5	52.0	47.4	46.2

In connection with the laboratory experiments described above, yeast cultivations were carried out on the full industrial scale at the "Activ" Yeast Factory of the Svenska Jästfabriks AB at Tomelilla, under the supervision of Mr. NORMANDER. The process was performed in a fermentation vat of 4.1 m cross-section and 4.8 m height, fitted with the usual air distributors and cooling coils. For the preparation of the potato juice, the potatoes were squeezed in a mash press with a perforated cylinder. 40–45 litres of juice was thus obtained from 100 kg potatoes. After precipitation of coagulable protein by acidification with sulphuric acid and centrifugation of the precipitate, the juice was added together with the wort in graduated quantities during the fermentation. Each cultivation was carried out with 2000 kg molasses and varying amounts of potato juice. Further details of the experiment and the results are set out in Table 34. On each occasion a control experiment was made without addition of potato juice.

Table 34

Yeast cultivation with molasses and addition of potato juice on the industrial scale.

Amount of molasses, kg	Amount of potato juice, litres	Amount of 25 % seed yeast, kg	Yeast yield, less seed yeast, calc. for 75 % water content	Percentage yield, calc. on molasses used (48 % sugar)	Water content of crop, %	Protein content of crop as % of dry weight
2000	—	588	1875	98.8	75.0	42.1
2000	450	588	2064	103.2	75.9	45.0
2000	—	602	1982	99.1	75.1	41.5
2000	900	602	2070	103.5	74.2	41.3
2000	—	603	1996	99.8	75.4	42.6
2000	1800	603	2082	104.1	75.9	45.4
2000	—	598	1970	98.5	74.2	42.7
2000	1800	598	2104	105.2	74.2	41.6

It will be seen that the results of the laboratory experiments with potato juice as supplementary material to molasses wort were confirmed on the full industrial scale.

The increased yields of yeast obtained by addition of potato juice to yeast cultivations on molasses are probably due to the growth factor content of the juice.

Chapter IV. Molasses as a substrate for *Saccharomyces cerevisiae*

Much has appeared in the literature concerning the relative qualities of different types of molasses as substrates for fermentation (CLAASSEN 1932, BRAUN and KOTSCHOPOULOS 1932, SPERBER 1945, AGARWAL, SINGH, KING and PETERSON (1947)). It has not been possible as yet, however, to give a satisfactory explanation for the differences observed. The purpose of the experiments described below was to contribute towards such an explanation.

When molasses is referred to in the present work, it always implies the waste product of the sugar industry which is utilised by the fermentation industry under the name "commercial molasses".

1. The chemical composition of molasses

As a consequence of the process employed in the winning of sugar from beet, almost all the components of the beet remain in the molasses that are left unchanged and unprecipitated during the refining and concentration of the raw juice. Further, the molasses contains materials formed in the manufacturing process, which pass through all stages without undergoing precipitation. Substances of the first group include a sugar residue, all inorganic salts, and certain organic non-sugar compounds, both nitrogen-containing and otherwise. The second group includes the degradation products of proteins and sugars, and the reaction products of amino acids and sugars, which MAILLARD (1912) named "melanoidines". For a review of the extensive recent literature treating this "Browning reaction" from the nutritional viewpoint, reference should be made to BARNES and KAUFMAN (1947).

a) Sugars

The sugar of a normal raw beet sugar molasses, apart from small amounts of invert sugar and raffinose, consists mainly of saccharose. In fermentation, invert sugar and saccharose are of equal value. The trisaccharide raffinose, on the other hand, can be utilised only to the extent of one third by *Saccharomyces cerevisiae*, since the saccharase of this yeast can only split this compound into fructose and the disaccharide melibiose. The latter is not fermented by *Saccharomyces cerevisiae*.

It is the practice in the sugar industry to refer to the "purity quotient" of a molasses, which means the ratio of the sugar content to the total solid content. This quantity gives a certain idea of the efficiency of the crystallisation in the factory. The higher the quotient is, the more the molasses loses the properties generally associated with its name and resembles a sugar syrup. It must, however, be observed that not only the quantity, but also the quality of the non-sugar material determines the crystallisability of a surup. The purity quotient is therefore not an accurate criterion of the efficiency of sugar extraction. The lower the purity quotient of a syrup is, the more it is to be regarded as a molasses. In the present account, when a molasses is considered with respect to its suitability as a substrate for yeast cultivation, its purity quotient will be recorded, since, as will be shown later, the ratio of sugar to non-sugar, or especially to nitrogen, appears to have a certain significance with respect to the yeast-forming capacity of a molasses.

b) Ash components

The ash content of raw beet sugar molasses, as carbonate ash, usually varies for swedish molasses between 8.5 and 11 %, having an average value of 9 %. Apart from certain trace elements, it consists largely of potassium and then, in order of decreasing occurrence, sodium, calcium, iron and magnesium. For the sugar worker the ash components are a disadvantage, in that they increase the solubility of the sugar, i.e. retard the crystallisation. The ratio of sugar to ash content of a molasses, the so-called salt quotient, provides a certain measure of the degree of crystallisation. For Swedish raw beet sugar molasses, the quotient for a well-extracted material is usually below 4.5. If the quotient has a value greater than 5, the crystallisation has been unsatisfactory. In the growth of yeast on molasses, however, the inorganic salts play a positive part, since they are utilisable by the yeast as essential materials, and contain trace elements which may stimulate the growth and influence both the fermenting power and protein content of the yeast. Mc HARGUE and CALFEE (1931) found, for example, that manganese increased the respiratory capacity and dry weight of yeast. ELVEHJEM (1931) found that the same applied to copper, and this was confirmed by Mc HARGUE and CALFEE.

Table 35 shows the compositions of the ashes of a Swedish beet sugar molasses and a Swedish refined molasses.[1]

It will be seen from the table that, in addition to the metals usually regarded as essential for plant nutrition, potassium, calcium, iron and magnesium, there are also a considerable number of trace elements which may be of great physiological importance.

Just as potassium plays an important rôle in the carbohydrate metabolism of animals, so it is actively involved in that of the yeast cell. PULVER and VERZÁR (1940) showed that in the very first phase of the fermentation (WILLSTÄTTER's (1937) period of polysaccharide formation) potassium is taken up by yeast cells. When the fermentation commences, i.e. when carbon dioxide begins to be evolved, the cells again release the potassium. Glycogen formation is thus associated with the uptake of potassium, and glycolysis with its liberation.

In the synthesis of yeast on molasses the potassium requirements of the yeast are covered by the high content of the molasses. Addition of potassium is thus without effect upon the yeast growth. However, in cases where the carbohydrate substrate is poor in potassium, as, for example, thick liquor, the first evaporation product in sugar manufacture, the yeast yield may be increased by addition of more potassium. The needs of the growing yeast cell for sodium, calcium and iron are covered in ordinary cultivation experiments by the molasses and also by the tap water. At all events, in the experiments described in the present communication, additions of these elements were without effect upon the yield. The magnesium content of the molasses is inadequate to satisfy the needs of the yeast. When the tap water is also poor in this element, it is thus essential to add more magnesium, say in the form of magnesium sulphate.

It is impossible to give precise instructions regarding the necessary amounts of inorganic salts. These must be ascertained for each individual case by trial cultivations.

c) Organic nitrogen-free non-sugar components

Very little information is to be found (even in the more recent literature) concerning the organic nitrogen-free compounds in molasses, other than sugar, that are of importance in the yeast synthesis. BEYTHIEN, PARCUS and TOLLENS (1889) and SCHÖNE and TOLLENS (1900) found 0.5 % lactic acid, ANDRLIK (1900) citric acid and HERZFELD (1901), working with spent molasses, detected up to 4.32 % lactic acid, 4.29 % acetic acid and 1.02 % formic acid, in addition to propionic, butyric, valeric and succinic acids and caramel substances, which were, however, not estimated quantitatively. More recent studies of unfermentable reducing constituents of molasses have been made by, among others, SATTLER (1948), ZERBAN (1943, 1946), ZERBAN and SATTLER (1942), ZERBAN and ERB (1947), SATTLER and ZERBAN (1945) and ERB and ZERBAN (1947).

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[1] The analysis was carried out by Professor H. LUNDEGÅRDH, of the Agricultural College in Uppsala, by the method of spectral analysis worked out by him for use in agricultural chemistry.

Ta

Qualitative and quantitative composition of the ash of Swedish raw beet sugar m
of the v

Tr denotes tra
For Cr and Ni, Sp indicates a concentration of less than 0.00003

	K	Na	Ca	Fe	Mg	Sr	Cu
Raw beet molasses.....	3.4	0.62	0.090	0.011	0.009	0.004	0.001
Refined beet molasses..	3.1	0.45	0.19	0.048	0.008	0.004	0.001

d) Nitrogenous components

Reports in the literature on the forms in which nitrogen occurs in molasses are chiefly written from the viewpoint of sugar technology, since nitrogen compounds influence in a high degree the crystallisability of the sugar and are thus marked molasses-stabilisers. These reports, both old and more recent, are nevertheless of importance in the study of the yeast-forming capacity of molasses, since organic nitrogen compounds, above all the amino acids, play a large part in the biological protein synthesis. BODENBENDER and IHLÉE (1880) discuss the various forms in which nitrogen is combined in molasses, distinguishing between ammonia nitrogen, amide and amino acid nitrogen, betaine and protein nitrogen. SMOLENSKI (1910) investigated the nitrogen compounds of Russian molasses. LIPPMANN (1885) detected glutamic acid, leucin and tyrosin in molasses. He supposed that these substances were liberated from the proteins of the beet in the course of the sugar manufacture as a consequence of the high alkalinity. STANEK (1912) found in molasses up to 2.8 % of glutamic acid, and believed that this, together with betaine, was the most important nitrogenous constituent, accounting for at least 20 % of the total nitrogen in the molasses. LIPPMANN (1896) investigated the nitrogenous components of beet juices and found in the extracted liquors from the sugar manufacture, which had been treated with lead acetate and phosphotungstic acid, and also sometimes with mercuric nitrate, the compounds xanthine, guanine, hypoxanthine, adenine, arginine, guanidine and allantoin. In his opinion the purine derivatives originated in nuclein compounds. Apart from the compounds mentioned above, asparagine, glutamine and their corresponding acids, betaine and choline, leucine, tyrosine, lecithin and legumin were detected in beet and beet juice. In 1903, EHRLICH studied the nitrogenous constituents of spent liquors from the sugar manufacture and found leucine and isoleucine.

Molasses also contains amines as split products from proteins. Trimethylamine occurs as a result of the breakdown of choline bases and betaine.

It will have become clear from the above account that molasses, in view of its content of a wide variety of organic nitrogen compounds, may be especially suitable as a substrate for growing yeast. When a molasses is being assessed with respect to organic nitrogen compounds, however, it must be borne in mind that these differ greatly in nutritive value for yeast. As has already been remarked, the assimilable nitrogen compounds differ greatly in their growth-promoting effects. It is therefore often difficult to decide whether the influence of a given compound is to be attributed

s and refined molasses. The quantities are expressed as percentages by weight
sses.

faint traces.
i less than 0.0005 % and for other elements less than 0.001 %.

In	Zn	B	Be	Cr	La	Li	Ni	Pb	Rb
0015	Tr	Tr	(Tr)	(Tr)	(Tr)	Tr	(Tr)	(Tr)	Tr
0015	0.005	(Tr)	(Tr)	Tr	0	Tr	(Tr)	Tr	Tr

to a growth-factor effect, or whether the compound has served as nutrition to the yeast.

The total nitrogen content of molasses varies greatly in amount and assimilability. This depends largely upon the climate and rainfall during the growth of the beet, and also upon the procedure in the sugar factory. The mode of fertilisation of the soil, upon which the beet is cultivated, and the variety of beet are also important factors. It is therefore unjustifiable to compare molasses from different countries without further consideration In Sweden, where the whole sugar beet production is directed by the Svenska Sockerfabriks AB, only one variety of beet is produced. Repeated soil analyses from the cultivated areas have made it possible to give the farmers advice as to the method of fertilisation. The raw material of the molasses, the sugar beet, should therefore be, in this country, of quite uniform character.

Table 36

Analyses of Swedish raw beet sugar molasses of the years 1935–1937.

Sugar factory	Polarisation %			Total nitrogen %			Carbonate ash %		
	1935	1936	1937	1935	1936	1937	1935	1936	1937
Arlöv	49.85	49.65	49.40	2.09	2.14	2.24	9.19	9.58	10.15
Hasslarp	51.20	50.85	51.50	2.00	2.02	1.89	9.93	9.73	10.41
Hälsingborg	48.95	49.85	49.80	2.06	2.21	1.85	9.65	9.82	10.49
Hököpinge	49.45	50.30	48.50	1.88	2.05	2.11	9.03	9.18	9.22
Jordberga	47.90	48.85	48.55	2.37	2.38	2.22	9.49	9.37	9.64
Karlshamn	49.15	48.25	48.40	1.92	1.84	1.85	9.93	9.59	9.95
Karpalund	48.70	48.80	47.75	1.73	1.88	1.95	9.29	9.19	9.28
Kävlinge	48.80	48.15	48.35	2.15	2.14	1.93	9.12	9.14	9.88
Köpingebro	48.30	48.15	48.50	2.06	2.17	1.87	8.97	8.94	9.62
Roma	49.00	48.40	48.60	1.98	2.05	1.81	9.71	8.46	9.64
Skivarp	48.75	48.45	48.40	2.20	2.10	2.12	9.01	9.20	10.16
Staffanstorp	49.20	49.85	50.80	2.17	2.13	2.14	9.56	10.05	10.34
Svedala	49.65	49.80	49.30	2.04	2.16	2.00	8.87	9.37	9.73
Säbyholm	49.00	48.65	48.50	2.02	2.04	1.94	9.90	9.81	10.12
Trelleborg	51.60	50.35	48.60	2.19	2.14	2.08	9.50	9.77	9.86
Örtofta	49.40	49.20	50.55	2.00	1.92	2.08	9.11	8.79	10.24
Mean value	49.31	49.22	49.09	2.05	2.09	2.01	9.39	9.37	9.92

In an annual report in the journal of the Svenska Sockerfabriks AB, "Förhandlingar vid Svenska Sockerfabriksdirigenternas Förenings årsmöte 1939", WIKLUND gives a summary of analyses of molasses from the campaigns of the years 1935–1937. In Table 36 we reproduce the values for polarisation, total nitrogen and ash content of the molasses from the various factories concerned.

The table shows that the total nitrogen content of the molasses varies greatly. In 1935 the maximum was 2.37 %, the minimum 1.73 % and the mean 2.09 %. The corresponding figures for 1936 were 2.38 %, 1.84 % and 2.09 %, and for 1937 2.24 %, 1.81 % and 2.01 %. Similar data are to be found in the literature for foreign molasses: SMOLENSKI (1910) for Russian, ANDRLIK, URBAN and STANĚK (1901) for Bohemian, HERZFELD (1925), STAIGER (1925, 1932), SPENGLER (1926), SPENGLER, PAAR and ZABLINSKY (1927), and WENDEL (1941) for German molasses.

It will be clear from the above remarks that molasses is a very heterogeneous material with respect to chemical composition, and is hence difficult to define from the chemical viewpoint.

2. Analyses of Swedish molasses from different campaigns. The effect on yeast growth of variations in composition of molasses

Experiments are described below which were carried out in an attempt to find analytically detectable characteristics of the molasses that might be correlated with the yields of yeast obtained from the respective materials. It was hoped in this way to make it possible to predict the yeast-forming capacity of a molasses.

During the campaign of 1944, 30 samples of molasses from 11 different factories were analysed and used in yeast cultivation experiments. The samples were taken from the factories as weekly average samples every third week during the campaign. They are therefore quite representative. In order to eliminate as much as possible any errors in the yeast yield due to the properties of the seed yeast, a control cultivation was carried out in each series with one and the same standard molasses. This molasses had given, in several cultivations carried out in different manners, a mean yeast yield of 51.2 g dry substance per 100 g polarising sugar. In the series of experiments with different molasses where the yield with this standard molasses was less than 50.0 or more than 52.0 g dry substance per 100 g polarising sugar, the results in the same series with the unknown molasses were rejected, since it was clear that in such cases the seed yeast had influenced the results excessively. The experiments were all carried out with Z III (cf. Chapter II, 6) as seed yeast, since this material had proved to be the most reliable with respect to yeast-forming capacity. The cultivations were otherwise carried out in the manner described for the standard experiment on p. 22.

In Table 37 are given the analytical values for the molasses and the yields obtained in the yeast cultivation experiments. The latter are arranged in order of decreasing magnitude.

The table gives the following information:

Column 1. Name of sugar factory, and sample.
» 2 a. Sugar content of molasses, determined by polarimetry.
» 2 b. Non-sugar content of molasses in per cent.
» 3. Dry weight of molasses in per cent, determined by saccharometer and expressed in degrees Balling.

Column 4. Total nitrogen of molasses in per cent, expressed for wet and dry material.
» 5. Amino nitrogen content of molasses in per cent, expressed for wet and dry material.
» 6. Amino nitrogen content of molasses in per cent of total nitrogen.
» 7. Sugar-nitrogen quotient of molasses $= \frac{\text{polarisation}}{\text{nitrogen}}$ (wet molasses).
» 8. Purity quotient of molasses $= \frac{\text{polarisation} \times 100}{\text{degrees Balling}}$.
» 9. Ash content of molasses, expressed as carbonate ash.
» 10. Salt quotient of molasses $= \frac{\text{polarisation}}{\text{ash}}$.
» 11. Yeast yield, in g dry substance per 100 g polarising sugar, obtained with the molasses in laboratory cultivations.

The analytical values in the table show a considerable variation in the sugar contents of the molasses between 45.2 and 49.6 %. The mean of all determinations was 47.25 %. The total nitrogen lay between 1.96 and 2.33 %, the mean being 2.11 %. The amino nitrogen content, as a percentage of the total nitrogen, varied in a high degree from 12.4 to 19.2 %, with a mean of 15.8 %. It should be noted, however, that these figures do not give the total amino nitrogen of the molasses, since e.g. the glutamic acid is present in the form of pyrrolidone carboxylic acid, and the amino nitrogen of this acid is not included in the van Slyke determination. The purity quotient lay between 59.6 and 64.7, with a mean of 59.7. The ash content varied from 8.5 to 10.1 %, with mean 9.6 %, and the salt quotient calculated from this and the polarisation value gave values between 4.5 and 5.8, with a mean of 4.9. The yields from the yeast cultivation experiments also varied considerably from 48.8 g to 53.5 g dry yeast substance per 100 g polarising sugar.

If the sugar content of the molasses is considered with relation to the yeast yield, it appears that the yield has a tendency to fall with increasing sugar content. There also seems to be a correlation between the sugar-nitrogen quotient and the yeast yield, the two quantities appearing to be in inverse proportion, i.e. a higher quotient corresponds to a lower yield. The analytical values in the table will later be subjected to statistical analysis.

In order to make a closer study of this correlation between the sugar-nitrogen quotient and the yield of yeast, experiments were carried out in which the quotient was altered by addition of extra sugar to the molasses. Yeast cultivations were then carried out with the altered molasses. The results are summarised in Table 38.

The results in the table show that the yield of yeast falls with increasing sugar-nitrogen quotient of the molasses. It is also possible to decrease the sugar-nitrogen ratio by precipitating sugar from the molasses as calcium saccharate. In such an experiment, when the untreated molasses gave 45.4 g dry yeast substance per 100 g polarising sugar, this yield was raised to 54.7 g per 100 g sugar when 14 % of the sugar had been precipitated.

The experimental results thus suggest that the ratio sugar: total nitrogen in the molasses is of importance in determining the yield of yeast. The nitrogen here concerned is the inherent non-ammonia nitrogen of the molasses, i.e. organic nitrogen, since the addition of further ammonia nitrogen, which brings about an alteration

Table 37

1	2 a	2 b	3	4		5		6	7	8	9	10	11
Molasses	Sugar content % polarisation	"Non-sugar" content %	Dry weight % °Balling	Total nitrogen %		Amino nitrogen %		Amino nitrogen in % of total nitrogen	Sugar : nitrogen quotient, pol./N	Purity quotient, pol. × 100/°Balling	Ash as carbonate %	Salt quotient pol./ash	Yeast yield in g dry substance per 100 g sugar
				wet	dry	wet	dry						
Jordberga I	45.2	30.5	75.7	2.25	2.97	0.35	0.46	15.5	20.1	59.7	9.8	4.6	53.5
Köpingebro II	46.2	31.3	77.5	2.31	2.98	0.40	0.52	17.4	20.0	59.6	9.9	4.7	53.4
Mörbylånga I	45.6	30.6	76.2	2.14	2.81	0.30	0.39	13.9	21.3	59.8	10.1	4.5	52.6
Jordberga II	46.4	29.1	75.5	2.18	2.89	0.34	0.45	15.6	21.3	61.4	9.5	4.9	52.2
Arlöv I	45.6	30.8	76.4	2.18	2.85	0.36	0.47	16.5	20.9	59.7	9.1	5.0	52.0
Mörbylånga II	46.0	30.4	76.4	2.01	2.63	0.29	0.38	14.4	22.9	60.2	10.1	4.6	51.8
Köpingebro I	46.6	30.3	76.9	2.22	2.89	0.36	0.47	16.3	21.0	60.6	9.9	4.7	51.7
Jordberga III	45.4	29.3	74.7	2.09	2.80	0.33	0.44	15.7	21.7	60.8	9.2	4.9	51.7
Svedala II	47.8	29.7	77.5	2.18	2.81	0.32	0.41	14.6	21.9	61.7	9.7	4.9	51.7
Svedala III	48.6	30.4	79.0	2.33	2.95	0.33	0.42	14.2	20.9	61.5	9.3	5.2	51.6
Roma I	46.4	28.6	75.0	2.07	2.76	0.31	0.41	14.9	22.4	61.9	9.0	5.1	51.5
Mörbylånga III	46.0	30.0	76.0	2.11	2.78	0.29	0.38	13.7	21.8	60.5	10.1	4.6	51.4
Karpalund III	48.6	27.0	75.6	2.10	2.78	0.36	0.48	17.3	23.1	64.3	9.7	5.0	51.4
Arlöv III	47.2	29.1	76.3	2.12	2.78	0.34	0.45	16.2	22.3	61.9	8.9	5.3	51.2
Roma II	48.2	27.0	75.2	2.09	2.78	0.32	0.43	15.5	23.1	64.1	8.5	5.7	51.1
Arlöv II	46.0	30.0	76.0	2.07	2.72	0.37	0.49	18.0	22.2	60.5	9.5	4.8	51.0
Svedala I	48.8	29.2	78.0	2.20	2.82	0.32	0.41	14.5	22.2	62.6	9.9	4.9	51.0
Säbyholm I	46.6	29.3	75.9	2.06	2.71	0.34	0.45	16.6	22.6	61.4	9.8	4.8	50.9
Karpalund II	48.8	28.9	77.7	2.05	2.64	0.35	0.45	17.0	23.8	62.8	10.1	4.8	50.9
Köpingebro III	48.0	29.5	77.5	2.10	2.71	0.40	0.52	19.2	22.9	61.9	9.3	5.2	50.6
Hököpinge I	49.4	27.0	76.4	2.12	2.77	0.35	0.46	16.6	23.3	64.7	8.8	5.6	50.6
Karlshamn II	47.8	29.3	77.1	2.10	2.72	0.34	0.44	16.2	22.8	62.0	10.3	4.6	50.4
Karpalund I	47.4	30.0	77.4	2.05	2.65	0.35	0.45	17.0	23.1	61.2	10.1	4.7	50.3
Linköping I	48.4	31.0	79.4	2.14	2.70	0.27	0.34	12.6	22.6	60.9	10.7	4.5	50.3
Säbyholm III	47.4	30.1	77.5	2.03	2.62	0.29	0.37	14.1	23.3	61.2	10.0	4.7	50.0
Säbyholm II	47.2	28.4	75.6	2.00	2.65	0.32	0.42	15.8	23.6	62.4	9.7	4.9	49.9
Karlshamn I	47.2	30.6	77.8	1.96	2.52	0.36	0.46	18.3	24.1	60.7	10.5	4.5	49.7
Hököpinge II	49.6	27.4	77.0	2.04	2.65	0.34	0.44	16.6	24.3	64.4	8.5	5.8	49.7
Linköping II	47.2	30.1	77.3	2.00	2.59	0.25	0.32	12.4	23.6	61.1	10.3	4.6	48.8
Mean value	47.2	29.5	76.7	2.11	2.75	0.33	0.43	15.7	22.4	61.6	9.7	4.9	51.1

Table 38

Yeast cultivation experiments with molasses and extra sugar.

Polarisation of molasses: 44.4 %.
Total nitrogen of molasses: 2.07 %.
Wort dilution: 1 : 38.
Seed yeast: 5 g Z III as 25 % yeast.
Cultivation process: cf. p. 22.

The stock wort for these experiments was made by dissolving 50 g molasses and 6 or 10 g sugar in 750 ml of tap water. Of this solution 297 ml was used for each cultivation, corresponding to 8.8, 11.2 or 12.8 g sugar. For a nitrogen content in the original molasses of 2.07 %, the sugar-nitrogen quotient is 21.4, or $\frac{44.4}{2.07}$.

For the molasses with additional sugar, the quotients are 27.2, or $\frac{56.4}{2.07}$ and 31.1, or $\frac{64.4}{2.07}$.

Composition of substrate. g molasses + sugar per cultivation	Sugar-nitrogen quotient	Yeast yield in g dry substance per 100 g sugar
19.8 g + 0 g sugar	21.4	51.6
19.8 g + 2.4 g »	27.2	50.9
19.8 g + 4 g »	31.1	45.3

in the sugar : nitrogen ratio, does not give, within certain limits, an increase in the yeast yield, but only an increased protein content of the yeast crop.

It is an interesting point that a mixture of a molasses producing a good yeast yield with a bad molasses, gives a better yield than would be expected from the proportions in which the two were mixed.

Table 39 gives the results of some yeast cultivation experiments on mixture of molasses of unequal value with respect to yeast-forming capacity.

It might therefore be expected that it would be possible to adjust the quantities of the organic and inorganic components so that these were optimal for yeast-forming capacity, when calculated upon the sugar content. For a closer study of this matter, yeast cultivation experiments were performed in which a material different from ordinary molasses, the so-called "thick liquor" of the sugar factory, was employed. To this extra organic and inorganic materials were added to bring the contents into the region of those of "good" molasses, i.e. molasses with low sugar-nitrogen and salt quotients.

Table 40 gives the chemical composition of a thick liquor, the first evaporated sugar concentrate in the manufacturing process.

It will be seen from the table that the thick liquor contains very little organic nitrogen, about 0.5 % calculated on the dry weight, and hence has an abnormally high sugar-nitrogen ratio. Yeast cultivation experiments were carried out with this material, the usual quantities of nutrient substances, ammonium sulphate, diammonium phosphate and ammonia being added. The low nitrogen content of the liquor was otherwise not taken into account. Simultaneously and in parallel with these experiments, cultivations were made in pure sugar solution, in a sugar solution made up according to the analysis of the thick liquor, and in a sugar solution composed according to the concept of a "good" molasses. In making up the last-named solution, it was assumed that a maximum of 50 % of the total nitrogen of an ordinary

Table 39

Yeast cultivation experiments on mixtures of molasses unequal in yeast-forming capacity.

Molasses A: pol. 46.4 %, total nitrogen 1.76 %.
Molasses B: pol. 58.8 %, total nitrogen 0.38 %.
Seed yeast: 5 g Z III as 25 % yeast.
Wort dilution: 1 : 35.
Cultivation process: cf. p. 22.

Molasses	Yeast yield in g dry substance per 100 g polarising sugar					
	Found				Average	
	I	II	III	IV	found	calculated additively
Molasses A	55.1	55.4	56.3	55.2	55.5	—
Molasses B	37.0	37.1	34.3	33.7	35.5	—
A+B 1 : 1	48.7	48.6	49.5	49.7	49.1	45.5
A+B 1 : 3	42.7	43.4	42.9	41.4	42.6	40.5

Table 40

Composition of a "thick liquor".

Polarisation	56.2 %
Degrees Balling	59.0
Total nitrogen in per cent of wet weight	0.29
Total nitrogen in per cent of dry weight	0.49
Amino nitrogen in per cent of wet weight	0.04
Amino nitrogen in per cent of dry weight	0.07
Amino nitrogen as percentage of total nitrogen	14.3
Percentage ash content as carbonate ash	1.10
Salt quotient	51.1
Sugar-nitrogen quotient	193.7

molasses is assimilable, and that the ash content, as carbonate ash, amounts to about 9 %. The assimilable organic nitrogen was added in the form of asparagine and the unassimilable nitrogen as betaine hydrochloride. The ash was represented by a mixture of potassium sulphate, magnesium sulphate, sodium chloride, calcium chloride and manganese sulphate. In all experiments ordinary tap water was employed. The seed yeast was Z III.

The experimental results are set out in Table 41.

The table shows that the pure sugar solution gave the lowest yield of yeast, only 14.6 g per 100 g sugar. The "adjusted" pure sugar solutions, however, gave appreciably higher yields, 25.5 and 46.4 g dry yeast substance respectively per 100 g sugar. The original thick liquor, giving 33.7 g dry yeast substance per 100 g sugar, occupied an intermediate position, since it gave a better yield than the pure sugar solution adjusted to the analytical content of the thick liquor, but a worse yield than the solution adjusted to the composition of a "good" molasses. The difference

Table 41

Yeast cultivation experiments with various original and adjusted sugar substrates.

Substrate	Sugar used in g per cultivation	Ash content of substrate in per cent	Salt quotient	Sugar-nitrogen quotient	Yeast yield, g dry weight per 100 g sugar	Protein content of yeast crop in per cent of dry weight
Thick liquor	10	1.1	51	194	33.7	41.7
Pure sugar solution	10	—	—	—	14.6	51.5
Sugar solution adjusted to composition of thick liquor	10	1.1	51	194	25.5	49.3
Sugar solution adjusted to composition of "good" molasses	10	9	5	17	46.4	47.5

between the thick liquor and the sugar solution adjusted to its composition may be due, among other things, to the presence in the former of other organic non-sugar components that contribute to the formation of yeast. It must also be taken into consideration that the thick liquor, as the first product of evaporation in the sugar manufacture, may be rich in factors of especial value in the yeast synthesis.

In Table 42 are collected the results of several experiments with adjusted molasses in which the salt quotient was regulated by addition of potassium sulphate and the sugar-nitrogen quotient by addition of organic and inorganic nitrogen compounds in accordance with the "good molasses" concept, without regard to the proportion of assimilable nitrogen in a "good molasses". In this manner the various substrates obtained an excess of assimilable nitrogen, which was reflected in high protein contents of the crops.

The stock substrates for the cultivation experiments were prepared as follows:

I. 40 g thick liquor per 750 ml ordinary tap water.
II. 40 g thick liquor and 4.4 g K_2SO_4 per 750 ml ordinary tap water.
III. 40 g thick liquor, 4.4 g K_2SO_4 and 5.65 g $(NH_4)_2SO_4$, corresponding to 1.20 g assimilable nitrogen, per 750 ml ordinary tap water.
IV. 40 g thick liquor, 4.4 g K_2SO_4 and 5.65 g asparagine, corresponding to 1.20 g assimilable nitrogen, per 750 ml ordinary tap water.
V. 40 g thick liquor, 4.4 g K_2SO_4 and 11.4 g aspartic acid, corresponding to 1.20 g assimilable nitrogen, per 750 ml ordinary tap water.

The stock worts prepared in this manner were added to each cultivation flask in graduated quantities totalling 297 ml. The cultivation was carried out in the way described on p. 22. From the ash content of the thick liquor, 1.10 %, and the polarisation value of 56.2 % sugar, the salt quotient for mixture I is calculated as 51, or $\frac{56.2}{1.10}$ and that for the other substrates II–V, after addition of 4.4 g K_2SO_4 per 40 g thick liquor, as 5.1, or $\frac{56.2}{11.1}$. The sugar-nitrogen quotient of the first and second mixtures was 194, or $\frac{56.2}{0.29}$ and that of the third, fourth and fifth mixtures, where

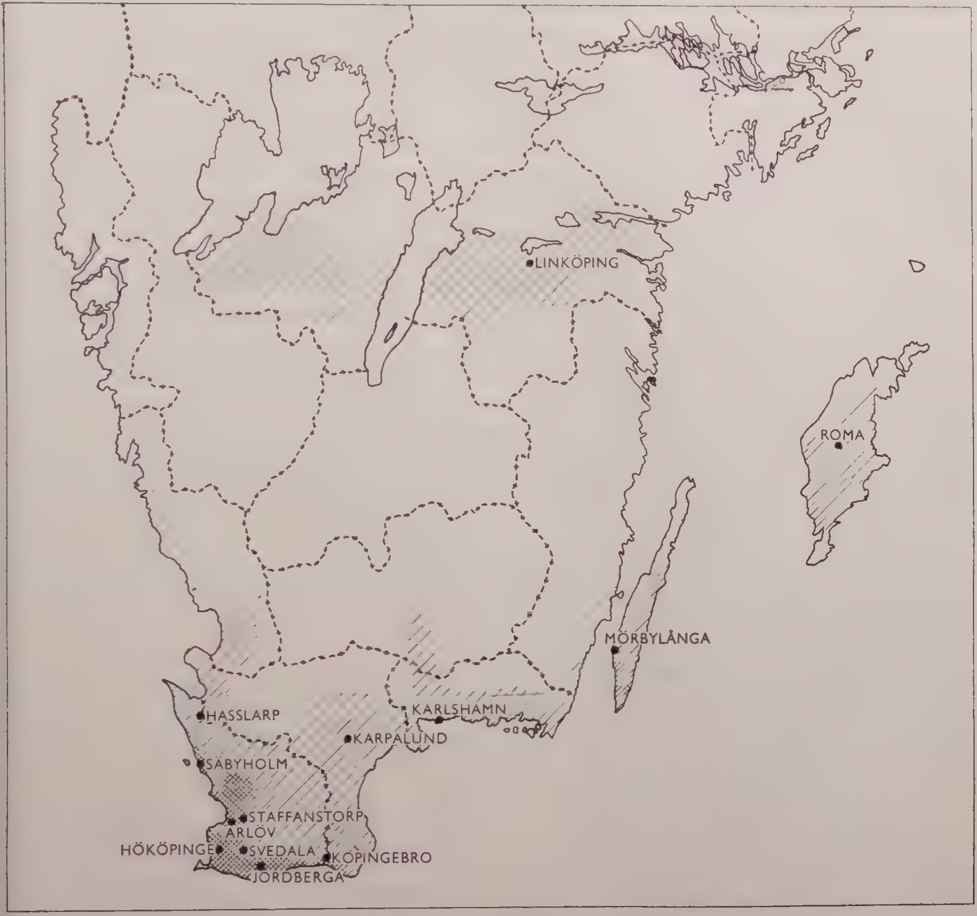
Table 42

Yeast cultivation experiments with "adjusted" thick liquors.

Substrate	Salt quotient	Sugar-nitrogen quotient	Yeast yield, less seed yeast, in g dry substance per 100 g sugar	Protein content of yeast crop in per cent of dry weight
I. Thick liquor	51	194	42.6	43
II. Thick liquor + K_2SO_4	5.1	194	43.6	40
III. Thick liquor + K_2SO_4 + $(NH_4)_2SO_4$	5.1	17	46.0	54
IV. Thick liquor + K_2SO_4 + asparagine	5.1	17	51.6	57
V. Thick liquor + K_2SO_4 + aspartic acid	5.1	17	57.2	53

a further 1.20 g nitrogen was added per 40 g thick liquor, i.e. per 22.5 g sugar, was 17, i.e. $\frac{22.5}{1.32}$.

If we consider the yield of yeast obtained with the unadjusted thick liquor, and compare it with the case where only potassium sulphate was added, we observe that these were not very different, whereas the yield and the protein content of the yeast cultivated on additional ammonium sulphate were greatly increased. If the inorganic nitrogen was replaced by organic, e.g. asparagine, a yeast of abnormally high protein content was obtained, but the yield was also appreciably raised. This indicates a stimulation of yeast growth by asparagine. It cannot be established on the basis of the present information whether this is to be interpreted as a growth-factor action of the asparagine or its utilisation by way of nutrition as a carbon compound. If aspartic acid was employed in the place of asparagine, a still higher yield of yeast was obtained under otherwise identical conditions. This yeast also had a high protein content, but lower than that obtained with asparagine. It seems that this may be explained by the fact that, as the amounts of asparagine and aspartic acid were calculated on the basis of their nitrogen contents and double the quantity of aspartic acid was hence necessary, the ratio of assimilable carbon to assimilable nitrogen was more favourable in the case of aspartic acid as more carbon was available. The experiments also showed that asparagine and aspartic acid can serve as carbon sources for growing yeast. This fact may throw some light upon the different effects of the various amino acids on the growth, and hence on the yield, of yeast. If it be imagined that the amino acids first partake in the yeast synthesis by undergoing deamination, and that the remaining structure forms a keto or a hydroxy acid, then this compound will be more or less fitted, in accordance with its configuration, for partaking, say by transamination, in the yeast synthesis. It is difficult to say which carbon compounds have this effect upon the yeast growth, but it may be suspected that they are those appearing in the aerobic carbohydrate breakdown. It must nevertheless be remembered that two α-amino groups cannot be considered as equivalent to one α-amino group and one amide group, since it is known from the work of Speck (1947) that for the conversion of glutamic acid to glutamine, energy is required in the form of adenosine-triphosphate. This should be reflected in the yield of the yeast. The higher protein content of the yeast in the experiment with asparagine probably indicates a greater activity of the asparagine in the protein



synthesis. Further experiments must, however, be made before it is possible to arrive at any definite conclusions in this respect.

The experiments described above confirm the tentative conclusion that the sugar-nitrogen quotient and the salt quotient of a molasses provide analytical criteria for the yeast-forming capacity of that molasses. Experiments will be described below which were carried out with the aim of providing further information concerning the significance of the sugar-nitrogen quotient. The materials used were molasses from 13 sugar factories from the campaign of 1945. The samples were analysed and used for yeast cultivation experiments in the laboratory. From 11 of the 13 factories three samples were obtained, representing the third, fifth and eighth weeks of the campaign. Two factories provided two samples each, from the third and sixth weeks. The raw sugar factories were so selected as to represent as great differences as possible in soil and climate. The factories were as follows: Hasslarp, Säbyholm, Staffanstorp, Arlöv and Hököpinge on the west coast of Scania, Svedala, Jordberga and Köpingebro on the south coast of Scania, Karpalund and Karlshamn further to the east, Roma on Gotland, Mörbylånga on Öland, and finally Linköping furthest to the north (cf. above map).

1		2 a	2 b	3	4		5		6	7	8	9	10	11
Molasses		Sugar content % polarisation	"Non-sugar" content %	Dry weight % °Ball.	Total nitrogen %		Amino nitrogen %		Amino nitrogen in % of total nitrogen	Sugar: nitrogen quotient, pol./N	Purity quotient, pol. × 100/°Ball.	Ash as carbonate %	Salt quotient pol./ash	Yeast yield in g dry substance per 100 g sugar
					wet	dry	wet	dry						
Karpalund	II	48.0	32.4	80.4	2.10	2.61	0.38	0.47	18.0	22.9	59.7	10.1	4.8	54.4
Karpalund	III	47.0	31.8	78.8	2.10	2.66	0.38	0.48	18.0	22.4	59.6	9.5	4.9	54.2
Jordberga	I	48.0	29.9	77.9	2.09	2.68	0.34	0.44	16.4	23.0	61.6	9.6	5.0	54.1
Köpingebro	III	48.2	30.3	78.5	2.08	2.65	0.40	0.51	19.2	23.2	61.4	9.3	5.2	53.9
Köpingebro	II	48.6	30.9	79.5	2.21	2.78	0.41	0.52	18.7	22.0	61.1	9.4	5.2	53.5
Karlshamn	III	47.4	30.7	78.1	2.11	2.70	0.33	0.42	15.6	22.5	60.7	9.7	4.9	53.5
Köpingebro	I	49.2	28.8	78.0	2.10	2.69	0.41	0.53	19.7	23.4	63.1	9.0	5.5	53.5
Jordberga	III	47.0	29.3	76.3	2.08	2.73	0.33	0.43	15.8	22.6	61.6	9.0	5.2	53.4
Staffanstorp	III	47.2	29.2	76.4	1.98	2.59	0.35	0.46	17.8	23.8	61.8	8.7	5.4	53.2
Hököpinge	I	50.2	32.1	82.3	2.23	2.71	0.42	0.51	18.8	22.5	61.0	9.4	5.3	52.8
Jordberga	I	48.0	29.9	77.9	2.09	2.68	0.34	0.44	16.4	23.0	61.6	9.6	5.0	52.7
Karpalund	I	49.8	30.7	80.5	1.97	2.45	0.38	0.47	19.2	25.3	61.9	10.5	4.7	52.6
Hököpinge	I	52.0	27.6	79.6	2.02	2.54	0.38	0.48	18.9	25.7	65.3	8.9	5.8	52.4
Karlshamn	I	48.0	29.7	77.7	1.97	2.54	0.27	0.35	13.8	24.4	61.8	10.3	4.7	52.2
Jordberga	II	45.8	29.9	75.7	2.04	2.69	0.34	0.45	16.7	22.5	60.5	9.2	5.0	52.1
Hököpinge	III	51.2	30.9	82.1	2.22	2.70	0.39	0.48	17.8	23.1	62.4	9.0	5.7	52.1
Arlöv	I	48.2	30.4	78.6	1.88	2.39	0.33	0.42	17.6	25.6	61.3	9.6	5.0	52.1
Säbyholm	I	48.0	31.6	79.6	1.86	2.34	0.29	0.36	15.4	25.8	60.3	10.2	4.7	52.1
Karlshamn	II	47.4	30.8	78.2	2.06	2.63	0.34	0.43	16.3	23.0	60.6	10.0	4.7	51.8
Arlöv	III	47.8	29.4	77.2	1.86	2.41	0.31	0.40	16.6	25.7	61.9	9.1	5.3	51.7
Mörbylånga	II	48.0	29.3	77.3	1.82	2.35	0.20	0.26	11.1	26.4	62.1	9.7	4.9	51.6
Staffanstorp	II	47.8	29.5	77.3	2.00	2.59	0.31	0.40	15.4	23.9	61.8	9.1	5.3	51.6
Arlöv	IV	48.0	28.2	76.2	1.89	2.48	0.33	0.43	17.3	25.4	63.0	8.7	5.5	51.6
Mörbylånga	I	49.0	28.4	77.4	1.85	2.39	0.28	0.36	15.1	26.5	63.3	9.9	4.9	51.4
Staffanstorp	I	48.2	28.3	76.5	1.79	2.34	0.30	0.39	16.7	26.9	63.0	9.9	4.9	51.4
Roma	II	47.2	29.2	76.4	1.97	2.58	0.26	0.34	13.2	24.0	61.8	9.0	5.2	51.1
Linköping	II	49.2	32.0	81.2	1.91	2.35	0.24	0.30	12.8	25.8	60.6	10.9	4.5	50.7
Hasslarp	II	52.2	31.3	83.5	2.00	2.40	0.33	0.40	16.7	26.1	62.5	10.4	5.0	50.7
Säbyholm	II	47.2	30.7	77.9	1.82	2.34	0.29	0.37	15.8	25.9	60.6	9.7	4.9	50.6
Hasslarp	III	52.8	30.9	83.7	2.00	2.39	0.30	0.36	15.1	26.4	63.1	9.9	5.3	50.6
Roma	III	48.0	28.0	76.0	1.73	2.28	0.26	0.34	14.9	27.7	63.2	8.8	5.5	50.6
Säbyholm	III	48.8	28.9	77.7	1.80	2.32	0.33	0.42	18.1	27.1	62.8	9.2	5.3	50.5
Linköping	I	51.0	30.4	81.4	1.81	2.22	0.24	0.29	13.1	28.2	62.7	11.1	4.6	50.0
Linköping	III	48.2	32.3	80.5	1.83	2.27	0.24	0.30	13.2	26.3	59.9	10.7	4.5	49.9
Arlöv	II	48.0	30.6	78.6	1.80	2.29	0.29	0.37	16.2	26.7	61.1	9.8	4.9	49.9
Hasslarp	I	53.4	29.5	82.9	1.76	2.12	0.30	0.36	17.0	30.3	64.4	10.5	5.1	49.9
Roma	I	51.2	25.0	76.2	1.72	2.26	0.24	0.31	13.7	29.8	67.2	8.7	5.9	49.5

The analytical values for the different molasses and the respective yields obtained in yeast cultivations in the laboratory are set out in Table 43.

It will be seen from the table that the sugar contents of the molasses varied considerably over the range 53.4 47 %. The mean value for all samples was 48.8 %. The total nitrogen lay between 2.23 and 1.72 %, with a mean of 1.96 %, calculated on the wet weight. The amino nitrogen, as a percentage of the total nitrogen, was found to be between 19.7 and 11.1 %, with a mean of 16.3 %. The purity quotient had values from 67.3 to 59.6, the mean being 61.9. The ash content varied between 11.1 and 8.7 %, mean 9.6 %. The salt quotient calculated from this and the polarisation value was from 5.9 to 4.5, with mean 5.1. The yields of yeast obtained in laboratory cultivation experiments with the various samples of molasses varied between 54.4 and 49.5 g dry yeast substance per 100 g polarising sugar.

If the yeast-forming capacity of the molasses is considered in relation to its sugar and nitrogen contents, it would seem that, for this season also, lower yields are associated with higher values of the sugar-nitrogen quotient. When the analytical values of the molasses from the campaign of 1944 are compared with those for 1945, they appear, on the whole, to be quite similar with similar ranges of variation. The molasses from the two campaigns differ, however, in one respect, namely in the relation of the absolute magnitude of the sugar-nitrogen quotient to the yield of yeast. For the campaign of 1944 the sugar-nitrogen quotient for the molasses varied between 20.0 and 24.3, and for 1945 between 22.0 and 29.8. If the molasses of the two campaigns are divided into three groups in accordance with their yields of yeast, and the average values of the sugar-nitrogen quotients for these groups are compared (Table 44), it will be seen that the quotient for 1945 is higher for the same yield than that for 1944. In other words, a given quotient does not correspond to the same yield from year to year. This fact cannot be explained on the basis of the available data. It nevertheless indicates that, apart from the ratio sugar : nitrogen, other factors also contribute to determining the yeast-forming capacity of a molasses. The sugar-nitrogen quotient may hence not be used as a sole criterion of yeast-forming capacity.

In order to establish whether there was a statistically significant correlation between the various analytically determined values and the yield of yeast, the relevant coefficients of correlation were calculated. These calculations were kindly performed by Dr. ÅSTRAND at the Statistical Bureau of the Svenska Sockerfabriks AB at Arlöv, to whom I wish to take this opportunity of expressing my most cordial thanks.

The coefficient of correlation is calculated from the formula

$$r = \frac{\Sigma\,(\triangle x \cdot \triangle y)}{\sqrt{\Sigma \triangle x^2 \cdot \triangle y^2}}$$

where x is the yield of yeast corresponding to the analytical value y. $(\triangle x \cdot \triangle y)$ is the product of the deviations of the corresponding means, while $(\triangle x)^2$ and $(\triangle y)^2$ are the squares of the individual deviations. If $r = 1$, the values of x give a straight line when plotted as functions of y. Here increasing values of x correspond to increases in y. If $r = -1$, the values also give a straight line, but increasing values of x then correspond to decreases in y. From the coefficients of correlation it may be established whether variations in the experimental data are associated one with the other.

In Table 45 the coefficients of correlation of the various analytical data with the yeast yield are shown. The statistical probabilities are represented as follows:

Table 44

Yields of yeast from molasses of different campaigns compared with sugar-nitrogen quotients of molasses.

Yield of yeast in g dry yeast substance per 100 g polarising sugar in molasses	Sugar-nitrogen quotient of molasses from campaign of	
	1944	1945
54.4 – 52.0	20.7	23.5
51.9 – 50.0	22.5	24.5
49.9 – 49.5	23.9	28.0

* indicates that the probability that the observed relationship is due to pure chance is < 0.05. ** denotes that this probability is < 0.01, and *** that it is < 0.001.

The calculations made as described above show a high degree of correlation between the nitrogen contents of the molasses of the two years with the yield of yeast, i.e. rising nitrogen content is associated with rising yield. The relation of the amino nitrogen to the yield is very different from one year to the other. The polarisation shows a weak correlation for the two years, but the remaining relationships may well be the results of chance.

A consideration of the quotients derived from the analytical data shows that there is no correlation between the ash content and the yield of yeast, but that there is a very definite correlation between the sugar-nitrogen quotient and the yield.

On the basis of the statistical analysis the relation between x and y may be represented by a straight line. This passes through the points of which the coordinates are the means of all corresponding values of x and y. The slope of the line may be derived from the so-called coefficient of regression. This indicates how much the yield of yeast will be increased by a change of one unit in the determining factor (the analytical value in question).

If y is expressed as a function of x, the coefficient of regression

$$R = \frac{\Sigma\,(\triangle x \cdot \triangle y)}{\Sigma\,(\triangle x^2)}$$

The relation between the yeast yield and the analytical data under consideration may be expressed by the equation $y = Rx + K$, which gives a straight line. K is a constant, expressing the value of y when $x = 0$. A calculation of the coefficient of regression by this means gives the values shown below in Table 46.

In Table 47 and Graph 7 are shown the correlations of polarisation and total nitrogen with the yield of yeast.

$$y\ (1944) = 69.2 - 0.808\,\frac{\text{pol.}}{\text{N}},$$

$$y\ (1945) = 64.9 - 0.520\,\frac{\text{pol.}}{\text{N}}.$$

Table 45

Mean values and standard deviations of data for molasses of the years 1944 and 1945. Correlation of these data with the yield of yeast.

	Year	pol.	Non-sugar content	Dry weight	Total nitrogen	Amino nitrogen	Carbonate ash	$\frac{\text{pol.}}{\text{N}}$	$\frac{\text{pol.}}{\text{Ash}}$	Yeast yield
n value	1944	47.23	29.48	76.71	2.11	0.33	9.67	22.4	4.90	51.13
	1945	48.85	29.98	78.82	1.96	0.32	9.66	25.1	5.07	51.89
ndard deviation . .	1944	2.6	4.1	1.5	4.3	10.4	5.9	5.1	7.2	2.11
	1945	3.7	5.0	2.8	7.3	17.6	7.0	8.3	6.9	2.6
elation with yeast ield	1944	−0.55**	+0.28	−0.30	+0.72***	+0.32	−0.11	−0.86***	−0.13	—
	1945	−0.38*	+0.25	−0.14	+0.76***	+0.70***	−0.25	−0.81***	+0.03	—

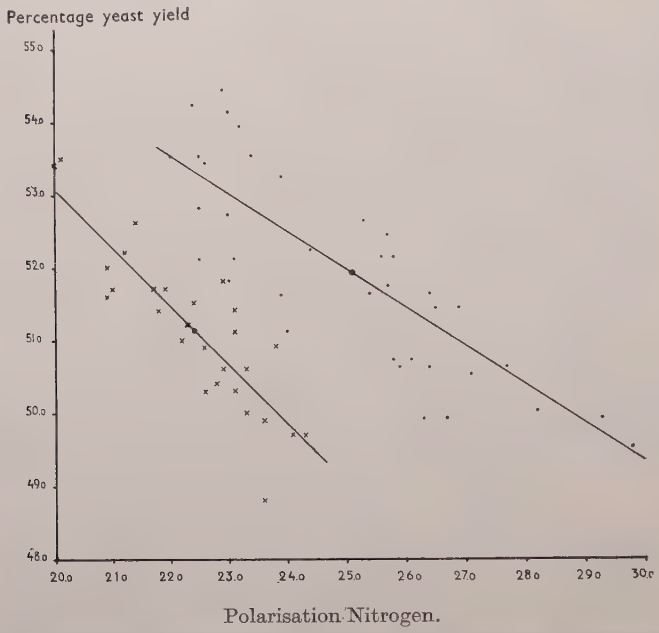
Table 46

Coefficient of regression on the yield of yeast of polarisation, total nitrogen and amino nitrogen of molasses and of the quotient $\frac{\text{pol.}}{\text{N}}$ (percentage increase in yeast yield associated with 1 % increase in total N, amino N and polarisation, and with increase in quotient $\frac{\text{pol.}}{\text{N}}$ by one unit).

	pol.	Total N	Amino N	$\frac{\text{pol.}}{\text{N}}$
1944	−0.47	8.5	9.9	−0.81
1945	−0.28	7.2	16.8	−0.52

Table 47

$\frac{\text{pol.}}{\text{N}}$	Yield of yeast	
	1944	1945
20.0	53.1	—
21.0	52.2	—
22.0	51.4	53.5
23.0	50.6	52.9
24.0	49.8	52.4
25.0	—	51.9
26.0	—	51.4
27.0	—	50.9
28.0	—	50.3
29.0	—	49.8
30.0	—	49.3



Graph 7. The correlations between yeast yield and the quotient Polarisation/Nitrogen are shown for the year 1944 in the left hand graph, and for the year 1945 in the right hand one.

The statistical treatment of the analytical data thus confirms the provisional interpretation of the experimental results, in that the yeast-forming capacity of a molasses rises with decreasing sugar-nitrogen quotient. It is also seen from the graph that the molasses from the years 1944 and 1945 were dependent upon factors other than the sugar-nitrogen quotient in respect of their yeast-forming capacities, since the relevant regression lines are not coincident, but lie one above the other.

In conclusion, it may be said that the results of the statistical analysis show that the sugar-nitrogen quotient of a given molasses may be used as a measure of the yeast-forming capacity of that molasses. It must, however, be observed that the quotient does not constitute an absolute standard, but may be used only for comparison of samples from one and the same year.

Chapter V

1. Summary

The present work is concerned with factors influencing the metabolic processes in growing yeast of the species *Saccharomyces cerevisiae* (top yeast) under aerobic conditions. Certain factors influencing the yeast growth have been investigated experimentally, especially with a view to ascertaining the conditions necessary for the maximum growth.

Chapter I

In this chapter a survey is made, based on previous literature, of the chemical processes involved in the biological degradation of carbohydrates in the presence of assimilable nitrogen under aerobic conditions.

1. It is explained at the outset that the amino acids, which may be regarded as the breakdown products of proteins, may also be regarded as the raw material from which the proteins are synthesised and may hence constitute one of the first stages in the protein synthesis in yeast. The formation of amino acids may be explained by the amination of substances formed during the carbohydrate degradation. The course of the carbohydrate breakdown is outlined in the form of simplified schemes to the extent necessary to explain the formation of a nitrogen-free basic material. Although the experimental work upon which these schemes are based has largely been carried out on animal material, they may nevertheless be regarded as valid in the main for yeast, since biochemical experience has shown that the enzymic processes in the yeast cell and in animal cells bear a great similarity.

The formation of the nitrogen-free substances of the yeast cell, the reserve carbohydrates, is discussed.

On the basis of the available literature, a survey is made of the known reactions concerned in the coupling of the aerobic carbohydrate degradation with the protein synthesis. Reference is made to the transamination reaction between keto acids and α-amino acids, which is considered in the more recent literature as being of especial importance in the protein synthesis. The rôle of the carbon dioxide constantly formed during the yeast synthesis is also discussed. Finally, a scheme is given for the coupling of the carbohydrate and amino acid turnovers with the protein synthesis.

2. No generally accepted theory exists for the biochemical reactions constituting the yeast synthesis. It follows that it is impossible to calculate a theoretical yield for the synthesis based upon stoichiometric relationships as, for example, can be done for the formation of alcohol from sugar by fermentation in the equation:

$$C_6H_{12}O_6 = 2\ C_2H_5OH + 2\ CO_2.$$

Experiments are described in the literature by EFFRONT, CLAASSEN, FINK and co-workers and BRAHMER aimed at the calculation of a stoichiometric yield and a maximum biological yield in the yeast synthesis. These are discussed, and the formulae given by the various authors for the calculation of the maximum yields of yeast, are presented. On the basis of our own experiments in the cultivation of yeast, carried out in the respirometer designed by SPERBER, calculations are made, according to BRAHMER's formula, of the maximum biological yield. The yields actually found were about 8 % lower than those attainable according to the formula.

Chapter II

Laboratory yeast cultivation experiments are described and the factors influencing the yield of yeast are discussed.

1. After a brief discussion of the apparatus described in the literature for yeast cultivations on the laboratory scale, the set-up employed in the present investiga-

tion is described. Two examples are given of actual yeast cultivations, which serve as models for the later experiments.

2. Yeast cultivation experiments made in SPERBER's respirometer are described. In these cultivations, carried out with saccharose as substrate, attempts were made to regulate the growth in such a manner that the respiratory quotient remained as near as possible to unity. Under such conditions reactions are avoided that lead to the breakdown of sugar to alcohol, and which render the carbohydrate no longer available for the yeast synthesis. During the cultivations the carbon dioxide evolved and oxygen consumed were measured. With the aid of analytical data for the seed yeast, the crop yeast and the residual substrate, carbon and nitrogen balance sheets for the cultivation experiments are drawn up.

Yeast cultivations were made under the same conditions with raw beet sugar molasses as substrate. The oxygen consumption in the synthesis of yeast on molasses proved to be higher than when saccharose was employed. This is attributed to the presence in the molasses of substances other than sugar which may influence the rate of the yeast growth. It also proved that the yield of yeast could be correlated with the oxygen consumption.

3. In the descriptions of yeast cultivation experiments in the literature, highly variable figures are given for the substrate concentrations employed, although regard is apparently not taken to the effect of such variation upon the yield. The term "substrate concentration" is here taken to mean the ratio of the amount of molasses employed to the final volume of the cultivation. Parallel and simultaneous yeast cultivations were carried out with different substrate concentrations, and these showed that the yield was influenced by the concentration. In general, greater dilution of substrate, within certain limits, gives rise to greater yields. Thus, in the consideration of the yield from a cultivation, account must be taken of the concentration of substrate employed. Experiments are comparable only when the concentrations have been the same.

4. The effect of pH upon the growth of yeast was investigated, both with synthetic substrate and with molasses as nutrient medium. The pH was maintained at a constant value by the addition of NaOH or H_2SO_4, and, in certain cases, by organic buffer materials. The experiments with molasses as substrate and NaOH and H_2SO_4 as pH-regulators, showed that the growth of yeast was independent of pH within the pH range of 4–6. In the experiments with organic buffer materials, the growth, i.e. the yield, of yeast, proved to decrease with increasing pH. This is ascribed to a toxic action of the buffer substances.

5. It is known from experience that the quality and quantity of the seed yeast are important factors in determining the yield of yeast from a cultivation. Up to the present, however, it has been impossible to give an explanation of this based upon experimental findings.

By means of cultivation experiments on saccharose with seed yeasts of different cultivation stages, a comparison was made of the yeast-forming capacities of the different yeasts. It was shown that it was possible to level out the differences between the yeasts to a large extent by the use of increased amounts of seed material.

Experiments are described in which the respiratory and fermentative capacities of the different seed yeasts were compared in the WARBURG apparatus. Although the respiration and fermentation curves of the different types distinctly differ from one another, these enzymic characteristics cannot be directly correlated with the respective yields of yeast.

Corresponding cultivation experiments with raw beet sugar molasses as substrate and yeasts from the different cultivation stages as seed materials, made it possible to draw valuable conclusions regarding the cause of the different yeast-forming capacities of the various yeasts. In the cultivations on molasses, as distinct from those on pure saccharose solution, the quantity of seed yeast was not so important in determining the yield. This is attributed to the fact that the molasses contains substances of value for yeast growth in sufficient quantities to make an increase in their amount through augmentation of the seed yeast without effect. Substances occurring both in yeast and in beet sugar molasses which are of importance for the growth of yeast, are amino acids and growth factors, i.e. certain vitamins. A survey is given of the investigations described in the literature which concern the effects of amino acids on the growth of yeast. An extensive account of the influence of growth factors is given in Chapter III.

The "acid-soluble" nitrogen of the various types of seed yeast, i.e. that nitrogen which can be extracted with trichloroacetic acid, was determined in an attempt to provide a criterion for the yeast-forming capacities of the different types. The results show that the acid-soluble nitrogen is of a certain importance for the growth, and hence the yield, of yeast, although the results do not permit any definite conclusions.

Chapter III

A study is made of the significance of organic growth factors in the growth of yeast.

1. A general review is made of the work in this sphere described in the literature.
2. The influence of growth factors on the growth of yeast was investigated under aerobic conditions. The growth factors studied were biotin, pantothenic acid, aneurine, pyridoxine and inositol. In the case of biotin, it was established that the yield of yeast is highly dependent upon the biotin content of the substrate. Furthermore, it was shown that the yield-increasing action of biotin reaches a maximum at very low concentrations. Of the other growth factors investigated, only aneurine and inositol showed a small effect upon the growth of yeast, while additions of pantothenic acid and pyridoxine were without effect under the conditions employed.

The biotin contents of yeasts from the different cultivation stages were determined. A microbiological growth method was used for these determinations, the test organism being *Neurospora crassa*. The biotin content of the yeasts cultivated anaerobically proved to be appreciably higher than that of the aerobic stages. In yeast cultivation experiments with yeasts from the different stages as seed materials, with and without additions of extra biotin, it was definitely established that differences in biotin content are largely responsible for the different yeast-forming capacities of the seed yeasts. By addition of biotin in cultivation experiments it is possible to equalize the yeast-forming capacities of the various seed yeasts.

The biotin contents of several molasses were determined. Significant differences were found in the contents of beet sugar molasses and cane sugar molasses. When a biotin-poor yeast was employed as seed yeast, it was possible, by addition of biotin, to increase the yield by several per cent.

The influence of a natural extract upon the growth of yeast, in this case potato juice, was studied. Experiments in the laboratory and on the factory scale showed that potato juice contains growth substances of importance for yeast growth. When the juice was added to yeast cultivations on molasses, the yield increased by several per cent.

Chapter IV

The properties of molasses are discussed, especially with respect to their chemical composition and the effect of variations in this upon the yeast growth.

A review is given of the literature concerning the chemical composition of molasses. On the basis of this review, the various constituents are considered with regard to their utilisation in the yeast synthesis. The fermentable sugar of molasses consists principally of saccharose. Reference is made to the literature concerning the unfermentable reducing substances of molasses. Of the non-sugar materials, the ash components are first considered, and detailed analyses are described of the ash of a Swedish raw beet sugar molasses and of a refined molasses. The organic nitrogen-free non-sugar constituents of molasses are briefly discussed.

Experiments are described which were carried out in an attempt to find certain analytical characteristics of molasses that might be correlated with the yields of yeast obtained with these materials. It was hoped in this manner to establish criteria for the yeast-forming value of molasses. The analytical data selected for this purpose were: sugar content, total nitrogen, amino nitrogen, dry weight and ash content. The data are treated statistically and certain significant correlations with the yeast yield are found. Of especial importance are the nitrogen content and the ratio sugar : nitrogen. The significance of the latter was shown in model experiments, were the ratio was altered by addition of sugar to the molasses.

It was shown in a series of experiments that when a good molasses, i.e. a material giving a good yeast yield, was mixed with an inferior molasses, better yields of yeast were obtained than were to be expected from the constitution of the mixture.

Experiments are described in which the yeast-forming capacity of an industrial sugar syrup, "thick liquor", was changed by addition of ash components and inorganic or organic nitrogenous materials. The results of these experiments support the finding that the sugar-nitrogen quotient provides an analytical index of the yeast-forming capacity of a molasses.

2. Conclusion

Discussion of the experimental results with respect to the factors determining the growth of *Saccharomyces cerevisiae* under aerobic conditions

The factors determining the growth of *Saccharomyces cerevisiae* may be concluded, on the basis of the experiments described here, to be largely biochemical in nature.

1. The reactions providing the basis of the growth of yeast on sugar and ammonia:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$
$$C_6H_{12}O_6 + NH_3 \rightarrow \text{protein} + CO_2$$

should be so regulated, that the amount of oxygen consumed is as equivalent as possible to the amount of carbon dioxide evolved. The respiratory quotient should thus be as close as possible to unity. A higher RQ corresponds to increased evolution of carbon dioxide, indicating alcohol formation ($C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$), which always involves a deterioration in the yield of yeast. By a properly regulated addition of sugar during the cultivation, the yeast synthesis may be so directed that the growth proceeds in accordance with the first two equations.

2. The method of yeast cultivation described in Chapter II, the respirometer method, makes it possible to work out a cultivation procedure providing the optimum conditions for yeast growth.

3. Apart from the actual cultivation procedure, the seed yeast, in respect of both its amount and its quality, plays a highly important part in determining the yeast growth. Increased quantities of seed yeast, within certain limits, give rise to higher yields. The content of growth factors (vitamins) in the seed yeast is the factor primarily determining its yeast-forming efficiency. The vitamin of importance to the strain of yeast employed in the present investigation is especially biotin, but aneurine and inositol are also not unimportant. The biotin-rich yeast cultivations hence provide the most efficient seed yeasts.

4. When molasses is used as substrate in yeast cultivations, its chemical composition is of importance. Of primary significance is the content of growth factors. The ratio of sugar to organic nitrogen is also a factor determining the yield. In general, it may be said that a molasses with a lower sugar content and a higher organic nitrogen content gives a better yield than a material with higher sugar and lower nitrogen.

5. The pH of the fermenting substrate is practically without influence on the growth of yeast over the range 4–6.

In addition to the biochemical factors mentioned above, which are of importance in determining the yield of yeast, two further factors, of a more technical character, may also be referred to, namely the dilution and the aeration of the wort. As far as the former is concerned, increased dilution of the wort gives, within certain limits, an improved yield of yeast. The aeration of the substrate should be so arranged that the carbon dioxide produced is largely removed. The aeration should also provide effective stirring, since otherwise alcohol formation will occur, with a consequent lowering effect upon the yield.

Appendix

Analytical methods

a) The yeast

Dry weight determinations

The content of dry substance was determined by drying the yeast sample at 105° C.

About 1 g of the yeast was spread out by means of a glass rod to a thin layer on the walls of a weighing bottle. A preliminary drying was carried out for two hours at 50° C. and a final drying for three hours at 105° C.

Ash determination

The ash content of yeast was determined by direct ashing of the sample.

About 2 g of the yeast was ashed in a previously ignited and weighed quartz crucible and ignited in a muffle furnace until the disappearance of all carbon. The crucible and contents were weighed after cooling in a desiccator.

Nitrogen determinations

1. *Total nitrogen*

Nitrogen was determined by the KJELDAHL method, in the modification of LUNDIN, ELLBURG and RIEHM (1935) and protein calculated as N × 6.25.

About 1 g of yeast was transferred to a 500 ml KJELDAHL flask with 10 ml 30 % hydrogen peroxide and about 0.5 g of copper sulphate or pure copper wire. After the yeast had been thoroughly wetted and the reaction had ceased, 20 ml of chemically pure concentrated sulphuric acid was added. The flask was heated until the sample blackened and SO_3 vapour began to form. A glass bulb was then placed in the neck of the flask and the heating continued until the sample became green, when about 15 g K_2SO_4 was added and the heating continued for 10 minutes. The combustion of organic material was then ended. After cooling, 160 ml of water was added. The ammonium sulphate formed was treated in the usual manner with 90 ml 35 % NaOH and the ammonia was distilled over into a receiver containing 20 ml 0.1 N–H_2SO_4. The unused sulphuric acid was titrated back with 0.1 N–NaOH and KJELDAHL indicator. The nitrogen was then calculated from the amount of sulphuric acid used. Multiplication by the factor 6.25 gave the protein content of the sample.

2. *Acid-soluble nitrogen*

In the present communication, the term "acid-soluble nitrogen" is taken to mean the nitrogen extractable with 8 % trichloroacetic acid from wet yeast. The nitrogen in the extract was estimated as described above.

About 10 g of wet yeast was stirred with 40 ml 8 % trichloroacetic acid and kept in the refrigerator for 4 hours. The mixture was then centrifuged. Nitrogen determinations were made on 10–15 ml portions of the clear centrifugate.

b) The fermented yeast-free wort

Nitrogen determinations

The total nitrogen was determined in an aliquot of the fermented wort by the same method as that given for the total nitrogen of yeast.

The determination of *the inorganic nitrogen* was carried out by distillation of an aliquot of the fermented wort with MgO.

25 ml of the fermented wort, freed from yeast, was diluted with 225 ml water in a KJELDAHL flask. About 5 g MgO was added and the liberated ammonia was distilled over into a receiver containing 0.1 N sulphuric acid. The nitrogen content of the wort was calculated from the amount of sulphuric acid used.

Alcohol determinations

The alcohol of the fermented wort was determined pyknometrically after distillation.

100 ml of the fermented wort, freed from yeast, was diluted with 50 ml water and distilled. The alcohol was determined pyknometrically in 100 ml of the distillate.

c) The molasses

Dry weight determinations

The accurate determination of moisture in sugar or sugar-containing materials by drying at 100–105° C. offers great difficulty. This is due, among other things, to the very hygroscopic nature of the products and to the retention of water by absorption. Certain sugars also show a considerable tendency to decompose at about 100° C., when volatile materials other than water are also evolved.

In the present investigation the dry weights of sugar-containing materials such as molasses were determined by specific gravity measurements. The technique employed was that usual in industry, namely the measurement of degrees BALLING with the saccharometer after "double dilution". By definition °Balling characterizes a saccharose solution containing the same percentage per weight as given by the Balling figure.

200 g molasses was diluted with distilled water in a beaker to a weight of exactly 400 g. After careful stirring, the molasses solution was poured into a glass cylinder and the dry weight was determined by means of a BALLING saccharometer (temperature correction). The dry weight of the original molasses was obtained by multiplication by two of the number of degrees BALLING read off on the instrument.

Sugar determinations

The sugar of molasses consists largely of saccharose, with small amounts of invert sugar and raffinose. Five different methods have been proposed for the determination of saccharose in molasses:

1. Polarimetric analysis directly, or after clearing of the molasses solution with lead acetate.
2. Determination of the sugar by means of a copper reduction method after inversion with hydrochloric acid.
3. Polarimetric determination of the saccharose by the method of CLERGET before and after inversion with hydrochloric acid.
4. Enzymic inversion, followed by polarimetric determination, after clearing the molasses solution with basic lead acetate and removal of lead from the filtrate with monoammonium phosphate.
5. Determination of the sugar by fermentation.

All these methods have the disadvantage that they are not absolutely specific for the sugar of the molasses that is utilisable in the cultivation of yeast. In the direct polarimetric analysis, for example, the presence of optically active amino acids, of raffinose and other optically active materials has a disturbing effect. As the majority of such substances are dextrorotatory, too high saccharose values are obtained. In order to eliminate these sources of error as much as possible, the proteins are usually precipitated before the polarimetric measurement with basic lead acetate and the excess lead is removed with monoammonium phosphate. This procedure, however, is not reliable, since raffinose and other optically active substances that may be produced during the manufacturing process by, say, overheating, are not precipitated. In the determination of saccharose by a copper reduction method, the preliminary inversion of the molasses with hydrochloric acid may convert other di- or polysaccharides present into reducing substances, so that an excessive saccharose content will be found. The same applies to the inversion method with subsequent polarimetry. Here the treatment of the sample with hydrochloric acid may bring about considerable changes in other optically active materials present. The enzymic inversion method, in which saccharase is used instead of acid, is also attended by some uncertainty, since the optical activity of the non-sugar materials may be altered by the action of the enzyme. The determination of saccharose by fermentation is very time-consuming. Furthermore, the alcohol value obtained after the fermentation does not give the actual content of alcohol-fermentable sugar in the sample, but a lower value, determined by the conditions of the experiment.

In cases where yeast cultivation experiments on molasses were carried out in the present investigation, the results of which were to form the basis of industrial procedure or were to be compared with results obtained on the industrial scale, the sugar determinations were however made by the polarimetric method of VENTZKE, which is normally employed in industry.

Exactly 13 g of molasses was dissolved in a little water in a 100 ml graduated flask. The proteins were precipitated with basic lead acetate, and the solution was made up to the mark. After thorough mixing, the solution was filtered and the optical rotation of the clear filtrate was measured in a 200 mm polarimeter tube. The reading on the scale, multiplied by two, gave directly the sugar content of the molasses.

In those cases where values of the highest accuracy were required for the alcohol-fermentable sugar of the molasses, the "yeast-desaccharification" method worked out by MENZINSKY (1943) was employed; see below.

Determination in molasses of sugar utilisable in the cultivation of yeast, by the "yeast-desaccharification method"

This method is based on the observation that if, to a solution containing sugar, an amount of yeast is added which is large in relation to the sugar present, and the solution is then shaken, the alcohol-fermentable sugar is removed from the medium in a very short time, while all other reducing materials are quantitatively unaffected.

When the reducing power of the solution is determined before and after the treatment with yeast, the content of assimilable (alcohol-fermentable) sugar may be calculated from the difference in these reducing powers.

Solutions

Enzyme solution

500 g compressed yeast is stirred with 25 ml ethyl acetate. After the yeast has been plasmolysed, 25 ml toluene is poured over it. The mixture is kept in a thermostat at 30° C. and stirred from time to time. After some days about 50 ml of the plasmolysed yeast suspension is diluted with 50 ml water and the yeast is centrifuged off. The clear centrifugate is used as the enzyme solution.

Protein-precipitating reagent

250 g mercuric sulphate is added, in small portions with shaking, to about 900 ml 10 % sulphuric acid in a one-litre graduated flask. When all the salt has dissolved, the solution is made up to the mark with 10 % sulphuric acid. The reagent is ready for use after filtration.

Sodium hydroxide solution

A sodium hydroxide solution is made of such a concentration (about 3.2 N) that when it is mixed with an approximately equal volume of mercury reagent in the presence of the sample, a pH of about 6 is obtained.

Analytical procedure

10 g of the molasses is diluted to 300 ml with ordinary tap water. 25 ml of this solution is adjusted to pH 5–5.5 with N–HCl. 10 ml enzyme solution is added and the inversion is allowed

to proceed in a thermostat at 30° C. for two hours. The proteins are then precipitated by simultaneous addition of mercury reagent and sodium hydroxide, the pH being maintained at about 6. As soon as the precipitate has become a pure yellow in colour, all the protein has been precipitated. The precipitate is filtered off and washed, the washings being added to the centrifugate. The solution is now made up to 250 ml and the excess of mercury is removed by the passage of H_2S. After the mercury sulphide has been filtered off, 100 ml of the filtrate is transferred to a conical flask and freed from hydrogen sulphide by the passage of air under gentle heating to about 40° C. The reducing substances in this solution are determined by a copper reduction method and calculated as invert sugar. 50 ml of the solution is placed in a 100 ml centrifuge tube and shaken with 5 g compressed yeast in the thermostat at 30° C. for 1 hour. The mixture is then centrifuged and the residual reducing material is estimated in the clear centrifugate by a copper reduction method. The difference between the sugar values before and after the treatment with yeast corresponds to the alcohol-fermentable sugar taken up by the yeast. The sugar content of the enzyme solution is determined in exactly the same manner, 25 ml of water being used instead of 25 ml of molasses solution. Then, by taking into account the dilutions and the sugar in the enzyme solution, it is possible to calculate the content of alcohol-fermentable sugar in the original sample of molasses.

Ash determinations

The ash was determined as sulphate ash after combustion of the sample with sulphuric acid. In accordance with the practice in the sugar industry, the ash is recorded as carbonate ash, this corresponding to 90 % of the sulphate ash content.

About 2 g of molasses was placed in a weighed quartz dish and thoroughly mixed with some drops of chemically pure sulphuric acid by means of a platinum wire. The wire was dried with a small piece of filter paper which was subsequently ignited with the sample. The dish was carefully heated until the sample had charred, after which it was ignited in a muffle furnace at about 500° C. (dull red heat) until the ash was pure white in colour. After cooling in a desiccator, the dish was weighed.

Nitrogen determinations

The total nitrogen was determined in the manner described above for the estimation of nitrogen in yeast. For each determination about 1 g of molasses was weighed out.

The amino nitrogen was estimated by the micro procedure developed by VAN SLYKE (1929). For the analysis 1 g molasses was diluted with distilled water to 100 ml. Of this solution 1 or 2 ml was employed, according to the suspected amino nitrogen content. The glutamic acid of the molasses was not included in the determination, since this occurs in the molasses in the form of pyrrolidone carboxylic acid.

d) Determination of the yield in yeast cultivations

In yeast cultivations, both on the laboratory and the industrial scale, the yield of yeast may be determined in several manners, for example by weighing a compressed sample of known water content, by counting the yeast cells in a known volume of suspension, by determining the amount of dry yeast substance in a known volume, or by measurement of the volume of a yeast suspension with known water or dry substance content. The choice of method is determined largely by the purpose for which the results are to be employed. The industrial worker, for instance, will immediately reject the counting method, since it is of little interest to him to know the number of yeast cells, large or small, present in one gram of yeast. He is largely concerned with the amount of yeast, in the form of moist compressed material, that has been obtained from a kilogram of raw material. In the more scientific consideration of yeast cultivations carried out under different conditions, the results of which are to be compared, variations in, say, the moisture content will be of great importance. All results must hence be calculated in terms of dry weight. The necessary determination may be made by direct weighing or indirectly by nephelometry. In the present investigation a direct determination was made in all experiments by weighing the dry yeast substance from a known volume of suspension. The yeast yield could then be calculated, when the total volume of the fermentation and the amount of seed yeast were known.

25 ml of the yeast suspension was pipetted into a 50 ml centrifuge tube and centrifuged. The clear wort was poured off and the yeast was stirred with water and centrifuged. This process was repeated. The yeast was then dried to constant weight at 105° C.

25 ml of the yeast suspension was filtered through a Jena glass filter crucible of type G 4 from Schott & Gen. When the yeast had been sucked off, the crucible was filled with water, which was sucked through. The washing was repeated once or twice. The yeast was dried at 105° C.

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(Abbreviations according to "List of Periodicals Abstracted", published by The American Chemical Society 1946).

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Optically active higher aliphatic compounds. VI

The synthesis of D(—)-21-methyltricosanoic, D(—)-21-methyltetracosanoic, and L(+)-21-methylpentacosanoic acids

By STINA STÄLLBERG-STENHAGEN

In order to get further information about the relation between optical activity and structure of methyl substituted long chain acids, which is important for the determination of the structure of naturally occurring acids of this type, it appeared desirable to prepare some acids with the active centre near the end of the chain opposite the carboxyl group. In the case of the shorter chain acids of this type resolved by LEVENE and MARKER (1), the carboxyl group is not distant enough from the active centre to be without influence on the optical rotation.

Optically active acids containing the methylethyl- end grouping have recently been found to occur in nature. In 1945 WEITKAMP (2) described the isolation of a series of dextrorotatory odd-numbered acids of this type from wool fat. VELICK and ENGLISH (3) have synthesized one of these so called ante-isoacids (WEITKAMP), *viz.* (+)-14-methylpalmitic acid, starting from active amyl alcohol derived from isoleucine. The relative configuration of the β-carbon atom of this amino acid is known (4), and it follows that the dextrorotatory higher ante-isoacids are sterically related to L-glyceraldehyde. (Using the nomenclature introduced in Part V (5), isoleucine may be written (+)-2(L)-amino-3(L)-methylpentanoic acid.) Optically active long chain acids having the end groupings methyl-*n*-propyl- and methyl-*n*-butyl- have not been reported previously.

The best initial material for synthesis of the types discussed appeared to be the appropriate active β-methyl substituted acids. According to LEVENE and MARKER (5), the resolution of racemic β-methyl substituted monocarboxylic acids is comparatively difficult, and it was therefore decided to investigate the possibility of preparing the active acids by mixed electrolysis of the appropriate enantiomorph of methyl hydrogen β-methylglutarate (6) and normal chain acid. The electrolysis is best performed in methanol solution (7). As the conditions near the electrodes cannot be controlled it was necessary, before applying the method to the present problem, to make sure that no reaction leading to a partially racemic β-methyl substituted acid takes place during the electrolysis.

It was found that active methyl hydrogen β-methylglutarate recovered from the reaction mixture after the electrolysis showed an optical rotation within the experimental error identical with that of the initial material. Electrolysis of L(+)-methyl hydrogen β-methylglutarate gives (+)-*methyl 3*(D),*6*(L)-*dimethylsuberate*, while the laevorotatory enantiomorph gives (−)-*methyl 3*(L),*6*(D)-*dimethylsuberate.*

The optical rotation of these esters obtained from different runs was consistently very close to α_{D}^{22} 10.65° (homogeneous; *l*, 1), and this in itself suggested that no racemization takes place during the electrolysis. Hydrolysis of the esters just mentioned gave the corresponding dibasic acids, which are well-crystallizing solid compounds. (+)-*3*(D),*6*(L)-*Dimethylsuberic acid* had $[\alpha]_{\mathrm{D}}^{22} + 16.68°$ (chloroform; *c*, 5.61) and (−)-*3*(L),*6*(D)-*dimethylsuberic acid* had $[\alpha]_{\mathrm{D}}^{23} - 16.63°$ (chloroform; *c*, 5.85). The active acids are dimorphic, the two forms melting at 81.5° and 84° respectively.

In case the enantiomorph of methyl hydrogen β-methylglutarate used in the electrolysis is not quite optically pure, or if racemization occurs during the reaction, the active methyl 3,6-dimethylsuberate will be contaminated mainly with *meso*-ester, but a very small amount of the enantiomorphic methyl 3,6-dimethylsuberate will also be present. In order to investigate whether impurity in the form of *meso*- and DL-acid respectively can be removed from active 3,6-dimethylsuberic acid by means of crystallization from solvents, the two inactive acids were prepared.

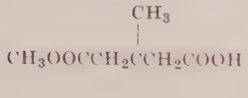
DL-*3,6-dimethylsuberic acid* was prepared by crystallization from a solution containing equal amounts of the enantiomorphs. The behaviour of the racemic acid is complicated by polymorphism. The acid appears to have a low melting form of m. p. 78°, and a high melting form of m. p. about 82°. meso-*3,6-Dimethylsuberic acid* was isolated by repeated crystallization of the mixture of DL- and *meso*-acid obtained by electrolysis of DL-methyl hydrogen β-methylglutarate. The *meso*-acid is less soluble than the racemic acid, and melts at 107.5–108.5°. There is evidence of polymorphism in this case also as previously melted material remelted at about 105°.

It was found in experiments in which about 5 % of *meso*- or of racemic acid were added to one of the enantiomorphs that the optical purity of the latter was restored by recrystallization. A specimen of (+)-methyl 3,6-dimethylsuberate was prepared from the optically pure enantiomorph, and its optical rotation was found to be α_{D}^{22} +10.73° (homogeneous; *l*, 1). As the corresponding figures for the rotation of esters obtained directly from the electrolysis are very close to this figure it can be concluded that no racemization occurs during the electrolysis.

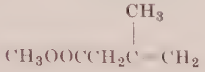
As a further check on the optical purity of active β-methyl substituted acids obtained by electrolysis, D(+)-3-methylundecanoic acid was synthesized by mixed electrolysis of L(+)-methyl hydrogen β-methylglutarate and *n*-octanoic acid. The dextrorotatory 3-methylundecanoic acid was obtained in an overall yield of 28 %, calculated on the active half-ester used, and its optical rotation was $[\alpha]_{\mathrm{D}}^{26} + 5.07°$, which is in excellent agreement with the figures found for the acid prepared by quite different routes in Part III (8), and by PROUT, CASON and INGERSOLL (9), *i.e.* $[\alpha]_{\mathrm{D}}^{22} + 5.06°$ and $[\alpha]_{\mathrm{D}}^{26} + 5.02°$ respectively. The method now described is by far the most convenient one for the preparation of this acid.

Mixed electrolysis of L(+)-methyl hydrogen β-methylglutarate and acetic acid gave D(−)-methyl 3-methylpentanoate in a yield of 32 % of the theoretical, calculated on the active half-ester used. Both during electrolysis of pure half-ester and in mixed electrolyses, a small fore-run boiling at 117–120° at atmospheric pressure was always obtained, and in the mixed electrolysis just referred to it was difficult to get a clean separation of this fore-run from the desired methyl ester. In order to be sure that a purification of the desired active 3-methylpentanoic acid could be effected at a later stage, the nature of the products contained in the fore-run was investigated.

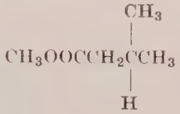
The electrolysis of a salt of a carboxylic acid usually gives a small amount of unsaturated hydrocarbon with one carbon atom less than the acid (cf. *e.g.* PETERsen (10)). The corresponding product from methyl hydrogen β-methylglutarate (XII) would be the methyl ester of 3-methyl $\Delta^{3:4}$-butenoic acid (XIII)


$CH_3OOCCH_2\underset{H}{\overset{CH_3}{C}}CH_2COOH$

XII


$CH_3OOCCH_2\overset{CH_3}{C}=CH_2$

XIII


$CH_3OOCCH_2\underset{H}{\overset{CH_3}{C}}CH_3$

XIV

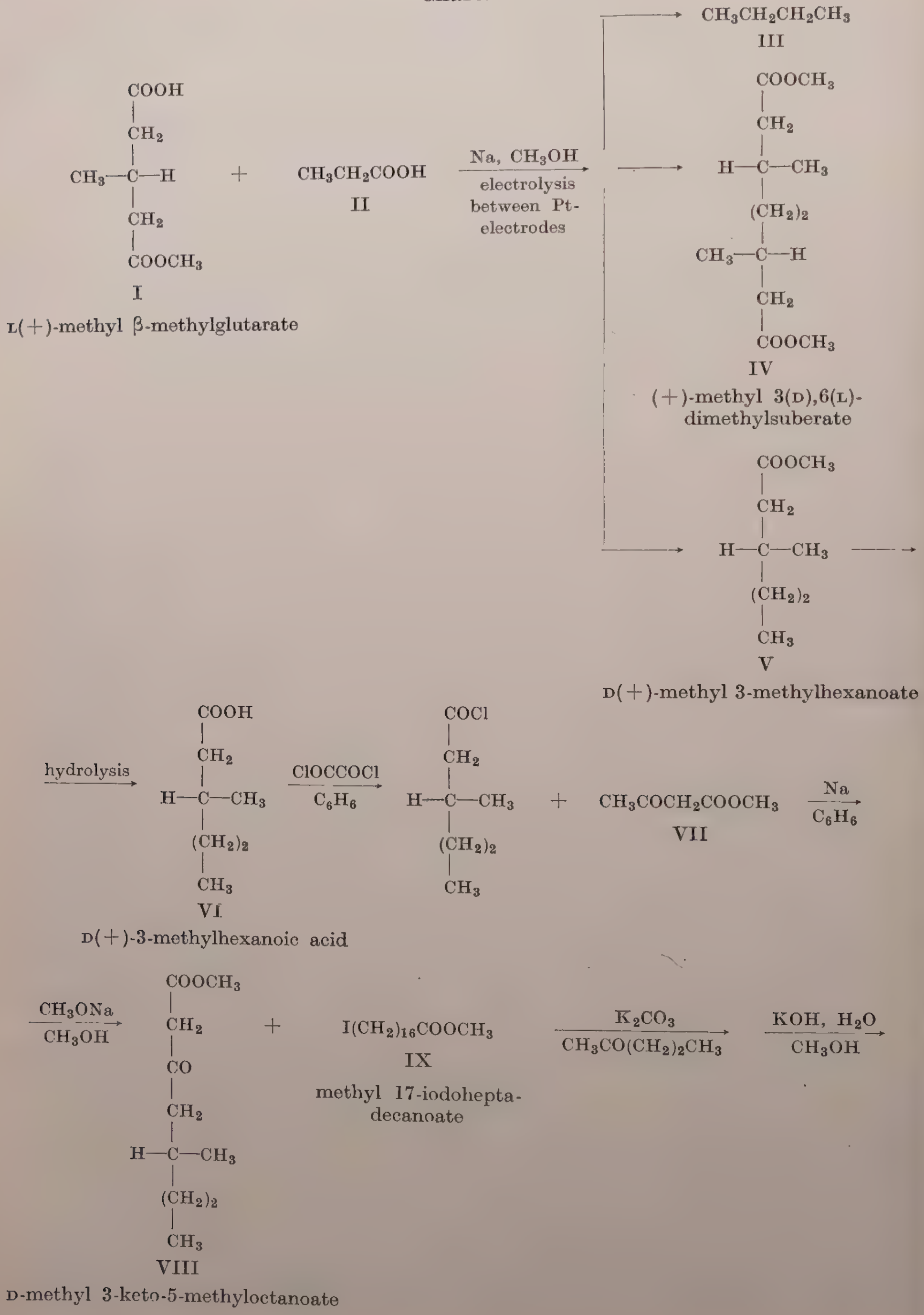
The analysis of the fore-run from the electrolysis of pure methyl hydrogen β-methylglutarate gave values for carbon and hydrogen corresponding to those for a mixture of an unsaturated and a saturated C_6 ester, the saturated part representing slightly more than half of the material. 3-Methyl $\Delta^{3:4}$-butenoic acid has been synthesized by Morton *et al.* (11), who found that it rearranges under the influence of alkali to β,β-dimethylacrylic acid. Hydrolysis of the ester mixture in the fore-run might therefore be expected to yield this acid together with a saturated C_5 acid. However, no solid β,β-dimethylacrylic acid could be isolated after hydrolysis, but after converting the liquid mixture of acids into the amides, *iso*valerylamide, m. p. 134.6–135.0°, was isolated and identified by comparison with an authentic specimen. Methyl *iso*valerate (XIV) is evidently produced by hydrogenation of (XIII) occurring at the platinum cathode surface during the electrolysis. β,β-Dimethylacrylamide melts at 65–66° (12), and its isolation could not be effected with the small amount of material available.

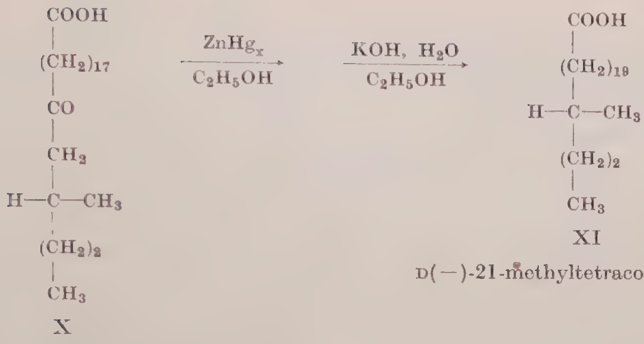
The D(−)-3-methylpentanoic acid obtained after hydrolysis of the methyl ester synthesized by mixed electrolysis had $[\alpha]_D^{23} -8.31°$ (homogeneous). This value is somewhat lower than the values previously recorded in the literature (cf. Table I). As the rotation of the (+)-methyl 3,6-dimethylsuberate obtained in the same run was $\alpha_D^{24} +10.60°$ (homogeneous; *l*, 1), indicating an amount of antipodal impurity of less than 0.5 %, this difference in rotation was evidently due to the presence of by-products from the electrolysis. In order to purify the acid it was converted into the amide, which after two crystallizations from benzene-light petroleum had m. p. 125.7–126.0°. van Romburgh (13) gives m. p. 124° and Marckwald and Nolda (14) m. p. 126.0°. It was later found that the addition of 5 % of *iso*valerylamide to D(−)-3-methylpentanamide lowered the m. p. of the latter by two degrees. It seems likely, therefore, that the C_5 amides present as impurity had been removed by recrystallization of the amide. D(−)-3-methylpentanoic acid obtained by hydrolysis of the amide had $[\alpha]_D^{23} +8.63°$. The remaining small difference between this figure and the figures collected from the literature (cf. Table I) is probably due to solvent and temperature effects.

The methyl esters of D(+)- and L(−)-3-methylhexanoic acid were obtained by mixed electrolysis of L(+)- and D(−)-methyl hydrogen β-methylglutarate respectively and propionic acid. The acids obtained after hydrolysis were, in this case also, purified via the amides, but it was found that the most constant boiling fractions obtained on distillation had the same optical rotation as the acids obtained by hydrolysis of the amides.

For L(−)-3-methylhexanoic acid Levene and Marker (5) give $[\alpha]_D^{27} -2.52°$, corresponding to a molecular rotation $[M]_D^{27} -3.28°$. In a later publication, however, the same authors (1) give $[M]_D^{25} -3.6°$, without giving any experimental details. The molecular rotation of the enantiomorphs synthesized via mixed electrolysis is

Chart.


I
L(+)-methyl β-methylglutarate
II
Na, CH₃OH
electrolysis between Pt-electrodes
III
IV
(+)-methyl 3(D),6(L)-dimethylsuberate
V
D(+)-methyl 3-methylhexanoate
hydrolysis
VI
D(+)-3-methylhexanoic acid
ClOCCOCl
C₆H₆
VII
Na
C₆H₆
CH₃ONa
CH₃OH
VIII
D-methyl 3-keto-5-methyloctanoate
IX
methyl 17-iodoheptadecanoate
K₂CO₃
CH₃CO(CH₂)₂CH₃
KOH, H₂O
CH₃OH



X

D(+)-19-keto-21-methyltetracosanoic acid

XI

D(−)-21-methyltetracosanoic acid

close to $[M]_D^{24}$ 3.17°, and it was found that the same molecular rotation was given by the specimen of laevorotatory acid described in Part III (8) after purification via the amide. The acid was in the latter case obtained by CLEMMENSEN reduction of L(+)-3-methyl-5-ketohexanoic acid, and had after distillation $[M]_D^{21}$ −3.9°. Purification via the amide was not thought necessary at the time, as the main interest was in the sign of the rotation.

The rotation found for L(−)-3-methylheptanoic acid prepared by mixed electrolysis of D(−)-methyl hydrogen β-methylglutarate and *n*-butanoic acid is lower than that reported by LEVENE and MARKER (5) for the acid obtained by resolution of the DL-acid. The author is unable to offer an explanation for the discrepancy, but in view of the results obtained for the homologous acids, it seems very unlikely that the value now obtained for the rotation will differ much from the correct value. The rotation of the (−)-methyl 3,6-dimethylsuberate obtained in the same run indicated the presence of less than 0.5 % of antipodal impurity.

The lengthening of the chain of the lower active β-methyl substituted acids was carried out by the β-keto ester method.

D(−)-3-Methylpentanoic acid, D(+)-3-methylhexanoic acid and L(−)-3-methylheptanoic acid were converted into the corresponding β-keto esters, *i.e.* D-*methyl 3-keto-5-methylheptanoate*, D-*methyl 3-keto-5-methyloctanoate*, and L-*methyl 3-keto-5-methylnonanoate* respectively, using the usual method (15), the yields being 56–70 % of the theoretical. The keto-enol equilibrium mixture obtained has in all cases a rotation of the same sign but smaller in magnitude than that of the acid used as initial material. The rotation of the β-keto ester will, of course, vary with the enol content.

Alkylation of the β-keto esters just mentioned with methyl 17-iodoheptadecanoate under usual conditions (cf. *e.g.* Part I (16)), followed by hydrolysis and ketonic cleavage of the alkylation products, gave D(−)-*19-keto-21-methyltricosanoic acid*, m. p. 76.9–77.1°, $[M]_D^{22}$ −7.5° (chloroform); D(+)-*19-keto-21-methyltetracosanoic acid* (X), m. p. 71.2–71.3°, $[M]_D^{22}$ +5.7°, and L(−)-*19-keto-21-methylpentacosanoic acid*, $[M]_D^{21}$ −6.6°, respectively. The last mentioned keto-acid has two crystalline forms with m. p. 64.2–64.6° and 66.3–66.7° respectively. The yields of the keto-acids were 63–68 % of the theoretical.

CLEMMENSEN reduction in ethanol solution, followed by hydrolysis of the ethyl ester formed, finally gave D(−)-*21-methyltricosanoic acid*, m. p. 67.8–68.1°, $[M]_D^{22}$ − 13°, D(−)-*21-methyltetracosanoic acid* (XI), m. p. 60.6–60.8°, $[M]_D^{21}$ −3.3°, and L(+)-

21-methylpentacosanoic acid, $[M]_D^{24} + 2.6°$. Like the corresponding keto-acid the C_{26} acid is dimorphous, with forms melting at 54.1° and 56.5–56.7° respectively.

For convenience, the complete synthetic route for D(−)-21-methyltetracosanoic acid is outlined in the chart above. The formulas for the optically active compounds are written according to FISCHER's convention (17, 18).

From the specific rotations given by WEITKAMP (2) for the C_{17} and C_{19} *anteiso*-acids from wool fat, their molecular rotations $[M]_D^{26}$ may be calculated to be +13.5° and +13.7° respectively. The rotations reported by WEITKAMP refer to measurements on acetone solutions. The low solubility in acetone of the C_{24} acid now prepared does not allow measurements to be carried out using this solvent, but the values for the molecular rotation of D(−)-21-methyltricosanoic acid in chloroform solutions are very close to those of the lower homologues just referred to.

Table I

Specific rotations for optically active lower β-methyl substituted acids

	Present investigation	From literature
D(−)-3-methylpentanoic acid	$[\alpha]_D^{23} - 8.63°$ (hom.)[1]	$[\alpha]_D^{17} - 8.98°$ (ligroin, *c*, 21.37) NEUBERG and REWALD (19)[2]
L(+)-3-methylpentanoic acid		$[\alpha]_D^{15} + 8.92°$ (hom.) v. ROMBURGH (13)[3] $[\alpha]_D + 8.37°$ (+8.86°)[5] (hom.) MARCKWALD and NOLDA (14)[3]
D(+)-3-methylhexanoic acid	$[\alpha]_D^{22} + 2.45°$ (hom.)[1]	
L(−)-3-methylhexanoic acid	$[\alpha]_D^{21} - 2.44$[4]; $[\alpha]_D^{24} - 2.43°$[1]	$[\alpha]_D^{27} - 2.52°$ (hom.) LEVENE and MARKER (5)[2]
L(−)-3-methylheptanoic acid	$[\alpha]_D^{21} - 4.00°$ (hom.)	$[\alpha]_D^{23} - 4.57°$ (hom.) LEVENE and MARKER (5)[2]

Experimental[6]

(−)-*Methyl 3*(L),*6*(D)-*dimethylsuberate.* The electrolysis was carried out in boiling methanol solution as described by RUZICKA *et al.* (7). Platinum electrodes were used, the electrodes and holder being those previously used by GUSTBÉE and STENHAGEN (20). Sodium (0.6 g) was dissolved in methanol (250 ml of A. R. quality (LKB-Produkter A.-B.), D(−)-methyl hydrogen β-methylglutarate (15 g, $\alpha_D^{19.8} - 2.76°$ (hom.; l, 4)) added, and the electrolysis carried out using a current of 4.5 Amps. After 40 minutes an additional amount of 10 g of the active half ester was added, and the electrolysis continued for 30 minutes. Methanol had to be added from time to time to replace loss due to evaporation. The reaction mixture was poured into water (500 ml), and the mixture extracted with three portions of ether. The combined ether extract was washed with sodium carbonate solution and with water. The ether solution was dried with sodium sulphate, the solvent removed, and the residue distilled, giving 10.3 g (57 % of the theoretical) of (−)-*methyl 3*(L),*6*(D)-*dimethylsuberate,* b. p. 94–96°, 0.8 mm.

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[1] Purified via the amide.

[2] By resolution of DL-acid.

[3] Derived from natural product.

[4] Prepared by reduction of L(+)-3-methyl-5-ketohexanoic acid (Part III) (8) and purified via the amide.

[5] Value obtained after correction for *iso*acid content.

[6] Melting points, optical rotations and crystal spacings were determined as described in Part I (16).

Optical rotation $\alpha_D^{22} - 10.59°$ (homogeneous; l, 1)
$[\alpha]_D^{22} - 10.68°$

Anal.: Calcd for $C_{12}H_{22}O_4$ (230.3) C, 62.57; H, 9.73 %
Found C, 62.66; H, 9.56 %

Immediately after the extraction of the neutral products, the sodium carbonate solution was acidified and extracted by means of ether. The solvent was removed and the residue distilled, giving 2.5 g of a colourless oil, b. p. 105–106°, 0.5 mm. The optical rotation of the product was $\alpha_D^{22} - 0.76°$ (hom.; l, 1). The rotation indicated the presence of some laevorotatory 3,6-dimethylsuberate. The specimen was therefore dissolved in a solution of sodium bicarbonate, and the solution extracted with four portions of ether. After acidification the active half-ester was taken up in ether. After removal of the solvent the residue was distilled, giving 1.0 g of D(−)-methyl hydrogen β-methylglutarate, $\alpha_D^{22} - 0.66°$ (hom.; l, 1). The rotation found is within the experimental error identical with that of the initial material at the same temperature.

(+)-*Methyl 3*(D),*6*(L)-*dimethylsuberate* was prepared in a manner analogous to that described for the antipode, using 45 g of L(+)-methyl hydrogen β-methylglutarate, $\alpha_D^{22.5} + 2.55°$ (hom.; l, 4). The half-ester was added in three portions. The yield of (+)-*methyl 3*(D),*6*(L)-*dimethylsuberate,* b. p. 98–99°, 0.7 mm, was 23.3 g or 72 % of the theoretical.

Optical rotation $\alpha_D^{22} + 10.67°$ (homogeneous; l, 1)

Anal.: Calcd for $C_{12}H_{22}O_4$ (230.3) C, 62.57; H, 9.63 %
Found C, 62.42; H, 9.60 %

(−)-*3*(L),*6*(D)-*Dimethylsuberic acid.* Hydrolysis of 3 g of the methyl ester gave, after crystallization from light petroleum (b. p. 60–80°)-benzene (about 3 : 1), 2.36 g of (−)-*3*(L),*6*(D)-*dimethylsuberic acid* in the form of long lustrous needles, m. p. 84.3–84.5°. Previously melted specimens showed the same m. p. A further recrystallized specimen showed m. p. 84.2–84.5°, but after previous melting the m. p. was 82°, indicating dimorphism.

Optical rotation $\alpha_D^{21} - 3.38°$ (chloroform; l, 1; c, 20.19)
$[\alpha]_D^{21} - 16.63°$ $[M]_D^{21} - 33.6°$
$\alpha_D^{23} - 1.95°$ (chloroform; l, 2; c, 5.85)
$[\alpha]_D^{23} - 16.6°$ $[M]_D^{23} - 33.6°$

Anal.: Calcd for $C_{10}H_{18}O_4$ (202.3) C, 59.83; H, 8.97 %
Found C, 59.64; H, 8.95 %

(+)-*3*(D),*6*(L)-*Dimethylsuberic acid.* Hydrolysis of 3 g of the methyl ester gave, after crystallization from light petroleum-benzene, 2.06 g of (+)-*3*(D),*6*(L)-*dimethylsuberic acid* in the form of long lustrous needles, m. p. 84.0–84.3°. After previous melting the m. p. was 81.2–81.6°.

Optical rotation $\alpha_D^{22} + 3.75°$ (chloroform; l, 4, c, 5.61)
$[\alpha]_D^{22} + 16.68°$ $[M]_D^{22} + 33.7°$

Anal.: Calcd for $C_{10}H_{18}O_4$ (202.3) C, 59.83; H, 8.97 %
Found C, 59.59; H, 9.03 %

DL-*3,6-Dimethylsuberic acid* was prepared by dissolving equal amounts of the enantiomorphs (0.4267 g of each) in benzene and adding light petroleum (b. p. 40–60°) until the DL-acid began to crystallize. The microcrystalline racemic acid thus obtained melted, on fairly rapid heating, at 78–79°. Previously melted material melted at 78–82°, but if the melt was rapidly solidified and immediately heated again, remelting occurred before 78°. This behaviour indicates dimorphism. The acid was obtained from acetone-light petroleum in the form of colourless prisms, but the thermal behaviour was unchanged.

meso-3,6-Dimethylsuberic acid. Electrolysis of DL-methyl hydrogen β-methylglutarate under the conditions described for the enantiomorphs gives a mixture of the methyl esters of DL- and *meso*-3,6-dimethylsuberic acid. The mixed esters (16 g) were hydrolyzed, and the *meso*-acid isolated by means of crystallization. After crystallization from benzene, water, benzene again, water, ethyl acetate and finally acetone, the *meso*-acid formed hard, transparent crystals melting at 107.5–108.5°. There was evidence of a lower melting modification formed on solidification of the melt, as remelting occurred at about 105°.

The *meso*-acid is less soluble in benzene than the DL-acid or the active acids. Both *meso*- and DL-acid are sparingly soluble in cold water. The racemic acid is very soluble in acetone at room temperature.

In order to find out whether a small amount of *meso*-acid could be removed from active 3,6-dimethylsuberic acid by recrystallization, a mixture containing 1.83 g of (+)-3,6-dimethylsuberic acid and 0.087 g of the *meso*-acid was recrystallized three times from benzene-light petroleum (b. p. 40–60°). The last crystallization gave mainly long prismatic needles but some white compact aggregates were also seen. The latter (15 mgs) were picked out, and both melting point (81.2–82.0°) and the optical rotation showed them to consist of the low melting form of the dextrorotatory acid. The needle-shaped crystals (1.30 g) consisted of the high melting form of the dextrorotatory acid, $[\alpha]_D^{22} + 16.6°$. It is thus possible to remove a small amount of *meso*-acid by means of crystallization.

An analogous experiment showed that a small amount of the antipode can also be removed by recrystallization. Laevorotatory methyl ester was added to a specimen of dextrorotatory ester, the rotation of the resulting specimen being $\alpha_D^{23} + 10.02°$ (hom.; l, 1). The partially racemic ester was hydrolyzed, and the acid recrystallized twice from light petroleum-benzene. The acid was obtained in the form of fine long needles, m. p. 84.0–84.3°. The optical rotation was $\alpha_D^{23} + 3.08°$ (chloroform; l, 1; c, 18.50) corresponding to a specific rotation $[\alpha]_D^{23} + 16.65°$. This rotation is within the experimental error identical with that recorded above.

(+)-Methyl 3(D)*,6*(L)*-dimethylsuberate obtained by esterification of the optically pure acid.* 2.68 g of the acid described above was esterified by means of methanol and sulphuric acid in the usual manner. Distillation in a micro-apparatus gave 2.36 g of methyl ester, b. p. 97°, 1 mm. d_4^{25} 0.989.

Optical rotation $\alpha_D^{25} + 10.66°$ (homogeneous; l, 1)
$[\alpha]_D^{25} + 10.76°$ $[M]_D^{25} + 24.78°$

The optical rotation α_D (hom.; *l*, 1) was found to decrease by 0.021 % per degree C increase in temperature over the range 17.6–33.0°. The corresponding density decrease was 0.09 % per degree C.

Attempts to determine the composition of the low-boiling fraction obtained in the electrolysis of methyl hydrogen β-methylglutarate. After electrolysis of 63 g of D(−)-methyl hydrogen β-methylglutarate, the fore-run boiling at 100–140° at atmospheric pressure was redistilled through a one metre Podbielniak column, giving 1.8 ml of b. p. 117–121°.

Anal.:	Found	C, 62.49; H, 9.65 %
	Calcd for $C_6H_{12}O_2$	C, 62.03; H, 10.41 %
	Calcd for $C_6H_{10}O_2$	C, 63.13; H, 8.83 %

The distillate evidently consists of a mixture of a saturated and an unsaturated ester, with the former slightly predominating. The ester mixture was hydrolyzed by means of 25 % potassium hydroxide in water solution. The liberated acids were distilled, but no solid product separated on cooling the distillate to low temperatures. The mixture of acids was therefore converted into the amides via the acid chlorides obtained by means of oxalyl chloride in hexane solution. The amides were extracted by means of ether using an apparatus for continuous extraction. After evaporation of the ether the solid crystalline residue was crystallized from benzene. After one crystallization the m. p. of the product was 128–129°, after two crystallizations 133–134°, and finally, after three crystallizations, the very thin lustrous plates obtained melted at 134.6–135.0°. Solidification took place at 134.4°. The product was identified as *iso*valeramide by comparison with an authentic specimen. The latter melted at 133.8–134.3°, and the m. p. was not depressed by admixture of the specimen derived from the by-products of the electrolysis. *Iso*valeramide from synthetic acid has been reported to melt at 135° (21). The two samples gave the same X-ray diffraction patterns.

D(+)-*3-Methylundecanoic acid.* L(+)-Methyl hydrogen β-methylglutarate (35.0 g of $\alpha_D^{28.8}$ +2.20° (hom.; *l*, 4)), *n*-octanoic acid (35 g, the commercial product (HEYL) was purified by distillation through a 1 metre glass packed column). 0.8 G of sodium and 250 ml of methanol were used, and the electrolysis performed in the manner described for the lower homologues. 4.9 Amps. were passed through during 3 hours. The reaction mixture was diluted with water, and the solution extracted by means of ether. The ether solution was washed with 5 % sodium carbonate solution followed by water. The ether was distilled off. A solution of potassium hydroxide (30 g) in water (20 ml) and ethanol (300 ml) was added to the residue, and the mixture refluxed for 5 hours. Part of the ethanol was removed under reduced pressure, water added, and the solution extracted by means of light petroleum (b. p. 40–60°) in order to remove the *n*-tetradecane formed during the electrolysis. The water-alcohol layer was acidified by means of hydrochloric acid and extracted by means of light petroleum (b. p. 40–60°). (+)-3,6-Dimethylsuberic acid is practically insoluble in light petroleum, and remains in the water-alcohol phase. The light petroleum layer, containing the active 3-methylundecanoic acid, was dried (sodium sulphate). The solvent was distilled off, and the residue on distillation gave 12.33 g (28 % of the theoretical) of D(+)-3-methylundecanoic acid, b. p. 138°, 3 mm.

Optical rotation α_D^{26} + 4.49° (homogeneous; *l*, 1)
$[\alpha]_D^{26}$ + 5.07°

The specific rotation given in Part III (8) for this acid was $[\alpha]_D^{22} + 5.06°$. Prout, Cason and Ingersoll (9) give $[\alpha]_D^{26} + 5.02°$.

D(−)-*Methyl 3-methylpentanoate.* Electrolysis of L(+)-methyl β-methylglutarate (35 g, $\alpha_D^{21.0} + 1.34°$ (homogeneous; *l*, 2)) and acetic acid (16 g (25 % excess), Kahlbaum "frei von höheren Homologen pro Analysi") was performed in a closed vessel fitted with a reflux condenser. The vessel was also cooled externally. 0.9 G of sodium and 300 ml of methanol were used, the half-ester and acetic acid being added in four portions. A current of 4.9 Amps. was passed through during 3.5 hours. The reaction mixture was poured into water, and the mixture extracted with three portions of ether. The combined ether solutions were washed with 5 % sodium carbonate solution, followed by water. After drying with sodium sulphate the solvent was distilled off, and the residue distilled through the 1 metre Podbielniak column.

I	128–135°,	756 mm	1.44 g	$\alpha_D^{24} - 3.03°$	(hom.; *l*, 1)
II	135–137.3°,	»	3.59 »	$\alpha_D^{24} - 5.45$	»
III	137.3–137.8°,	»	6.64 »	$\alpha_D^{24} - 6.01$	»
IV	137.8–87°,	756–0.4 mm	1.6 »	$\alpha_D^{24} - 4.94$	»
V	87°,	0.4 mm	1.11 »	$\alpha_D^{24} + 5.00°$	(hom.; *l*, 0.5)
VI	87°,	0.4 »	5.0 »	$\alpha_D^{24} + 10.60°$	(hom.; *l*, 1)

Redistillation of the fractions I, II, and IV gave 2.70 g of b. p. 137.3–138.0°, 756 mm, $\alpha_D^{23} - 6.00°$ (hom.; *l*, 1). The total yield of D(−)-*methyl 3-methylpentanoate* was thus 9.34 g or 32.5 % of the theoretical, calculated on the active half-ester used.

Optical rotation $\alpha_D^{24} - 6.01°$ (homogeneous; *l*, 1)

D(−)-*3-Methylpentanoic acid.* Hydrolysis of the methyl ester just described (9 g) gave 7.32 g of D(−)-3-methylpentanoic acid, b. p. 90°, 10 mm, $\alpha_D^{23} - 7.68°$ (hom.; *l*, 1). After purification via the amide described below, the acid showed d_4^{24} 0.924 and an

Optical rotation $\alpha_D^{23} - 7.97°$ (homogeneous; *l*, 1)
$[\alpha]_D^{23} - 8.63°$ $[M]_D^{23} - 10.03°$

D(−)-*3-Methylpentanamide.* 7.2 G of the acid was converted into the acid chloride by means of oxalyl chloride (8 ml) in benzene solution (25 ml). The mixture was kept at a temperature of 40° for 4 hours. Excess oxalyl chloride and part of the benzene were distilled off at atmospheric pressure through a Weston type vacuum-jacketed column. The residue was cooled to room temperature, and poured into an excess of cold (+5°) aqueous ammonia. The mixture was shaken vigorously for 5 minutes. Excess dilute hydrochloric acid was added, and the amide extracted by means of ether, using an apparatus for continuous extraction. The amide was crystallized from light petroleum (b. p. 95–100°), giving 5.39 g in the form of lustrous plates. M. p. 123.2–124.0°. Recrystallization from light petroleum-benzene (10 : 1) gave 4.4 g in the form of very beautiful thin lustrous plates of m. p. 125.7–126.0°. For the antipode van Romburgh (13) gives m. p. 124° and Marckwald and Nolda (14) m. p. 126.0°.

Optical rotation $\alpha_D^{23} - 1.47°$ (chloroform; *l*, 4; *c*, 4.96)
$[\alpha]_D^{23} - 7.42°$ $[M]_D^{23} - 8.53°$

D(+)-*Methyl-3-methylhexanoate* (V). The electrolysis was carried out using L(+)-methyl hydrogen β-methylglutarate (I) (47 g, $\alpha_D^{22.5}$ + 2.55° (hom.; *l*, 4)), and propionic acid (23 g, prepared from ethanol via ethyl iodide and nitrile synthesis). 0.8 G of sodium was dissolved in methanol (250 ml), and the mixture of half-ester and propionic acid added in six portions, 4.8 Amps. being passed through for a total time of 2.5 hours. The reaction mixture was worked up in the manner described for the lower homologue. Distillation through a 1 metre Podbielniak column gave the following fractions.

I	52—60°, 60 mm	1 ml		
II	60—87.8°, »	2 ml		
III	87.8—88.0°, »	9.34 g	α_D^{23} + 1.22°	(hom.; *l*, 1)
IV	88—93°, 60—0.5 mm	1.5 ml		
V	93°, 0.5 mm	8.0 g	$\alpha_D^{22.5}$ + 10.50°	»

Fractions II and IV were obtained and redistilled through the same column, giving 1.8 g of b. p. 88°, 60 mm, α_D^{23} + 1.20° (hom.; *l*, 1). Total yield of D(+)-*methyl 3-methylhexanoate* (V) 11.14 g or 26 % of the theoretical.

In a second experiment the main ester-fraction had α_D^{21} + 1.23° (homogeneous; *l*, 1) and the high-boiling fraction (dextrorotatory 3,6-dimethylsuberate (IV)) α_D^{23} + 10.62°.

d_4^{23} 0.873. A specimen of ester prepared from acid purified via the amide showed an

Optical rotation α_D^{23} + 1.24° (homogeneous; *l*, 1)
$[\alpha]_D^{23}$ + 1.42° $[M]_D^{23}$ + 2.05°

Anal.: Calcd for $C_8H_{16}O_2$ (144.2) C, 66.63; H, 11.18 %
Found C, 66.43; H, 11.13 %

D(+)-3-*Methylhexanoic acid* (VI). Hydrolysis of 10.5 g of the methyl ester gave the free acid which was distilled through the 1 metre Podbielniak column. The acid distilled at 96°, 4 mm. The following cuts were taken

I	2.64 g	α_D^{23} + 2.16°	(homogeneous; *l*, 1)
II	4.66 »	α_D^{23} + 2.18°	»
III	1.29 »	α_D^{23} + 2.16°	»

Total yield 8.59 g or 91 % of the theoretical, calculated on the methyl ester. After purification via the amide the acid had an

Optical rotation α_D^{23} + 2.22° (homogeneous; *l*, 1)
$[\alpha]_D^{23}$ + 2.43° $[M]_D^{23}$ + 3.16°

Anal.: Calcd for $C_7H_{14}O_2$ (130.2) C, 64.58; H, 10.84 %
Found C, 64.63; H, 10.75 %

D(+)-3-*Methylhexanamide.* The amide was prepared via the acid chloride. After crystallization from light petroleum (b. p. 40–60°) the amide formed large, thin lustrous plates, m. p. 90.5–91.2°.

Optical rotation $\alpha_D^{21} + 0.16°$ (chloroform; *l*, 2; *c*, 4.11)
$[\alpha]_D^{21} + 2.0°$ $[M]_D^{21} + 2.5°$
$\alpha_D^{21} + 0.36°$ (75 % ethanol; *l*, 1; *c*, 7.23)
$[\alpha]_D^{21} + 5.0°$ $[M]_D^{21} + 6.4°$

Anal.: Calcd for $C_7H_{15}ON$ (129.2) N, 10.84 %
Found N, 10.80 %

L(−)-*3-Methylhexanoic acid* from methyl ester obtained by mixed electrolysis of D(−)-methyl β-methylglutarate ($\alpha_D^{21} - 2.65°$ (hom.; *l*, 4)), and propionic acid. The experiment was carried out as described for the antipode, and gave L(−)-methyl 3-methylhexanoate in 30 % yield. The laevorotatory 3,6-dimethylsuberate simultaneously formed had $\alpha_D^{22.5} - 10.58°$ (hom.; *l*, 1). After hydrolysis of the methyl ester the acid was distilled through a 50 cm Podbielniak column, and finally redistilled through a 1 metre column of the same type. The following fractions were collected in the latter distillation

I	100.3–102.0°,	5 mm	1.6 ml	$\alpha_D^{23} - 1.82°$	(hom.; *l*, 1)
II	102.0–102.6°,	5 »	1.5 »	$\alpha_D^{23} - 2.05°$	»
III	102.6–102.7°,	5 »	2.0 »	$\alpha_D^{23} - 2.17°$	»
IV	102.7°,	5 »	1.5 »	$\alpha_D^{23} - 2.24°$	»
V	102.7°,	5 »	1 »	$\alpha_D^{23} - 2.24°$	»

The rotations of the fraction IV and V are identical with that shown by acid purified via the amide.

L(−)-*3-Methylhexanamide* from acid obtained by reduction of L(+)-3-methyl-5-ketohexanoic acid. The specimen of reduced acid described in Part III (8) had been purified by distillation only. The optical rotation reported was $[M]_D^{21} - 3.9°$. 1.55 g of this preparation was converted into the *amide* in the usual manner. After crystallization from light petroleum (b. p. 60–80°), 0.95 g of the amide was obtained in the form of lustrous plates, m. p. 90.7–91.2°. After further recrystallization from the same solvent the m. p. was 90.9–91.4°.

Optical rotation $\alpha_D^{22} - 0.20°$ (chloroform; *l*, 2; *c*, 5.03)
$[\alpha]_D^{22} - 2.0°$ $[M]_D^{22} - 2.6°$

Anal.: Calcd for $C_7H_{15}ON$ (129.2) N, 10.84 %
Found N, 10.73 %

L(−)-*3-Methylhexanoic acid* by reduction of L(+)-3-methyl-5-ketohexanoic acid. The amide just described (0.82 g) was hydrolyzed by refluxing for 24 hours with potassium hydroxide (5 g) and water (25 ml). The alkaline solution was acidified, and the acid taken up in ether. After the ether solution had been washed with water and dried with sodium sulphate, the solvent was removed, and the acid distilled in a micro-apparatus. B. p. 93°, 5 mm. Yield 0.56 g of colourless liquid. d_4^{22} 0.913.

Optical rotation $\alpha_D^{22} - 2.23°$ (homogeneous; *l*, 1)
$[\alpha]_D^{22} - 2.44°$ $[M]_D^{22} - 3.18°$

L(−)-*Methyl 3-methylheptanoate.* The electrolysis was carried out as described for D(−)-methyl 3-methylpentanoate, using 40 g of D(−)-methyl hydrogen β-methylglutarate ($\alpha_D^{22.4}$ −2.60° (homogeneous; *l*, 4)), 26 g of *n*-butanoic acid, 0.8 g of sodium and 300 ml methanol. The butanoic acid used was prepared from ethanol via malonic ester synthesis. The intermediate ethylmalonic acid was crystallized twice from a mixture of benzene and light petroleum (b. p. 40–60°) containing a small amount of ethanol. M. p. 112–112.5°. The value found in the literature is 111.5° (22).

4.8 Amps. were passed through during 3.5 hours. The neutral products from the electrolysis were collected in the manner already described. Distillation was carried out using the 1 metre Podbielniak column.

I	up to 75°,	30 mm	1 ml		
II	75–90.2°,	30 »	0.85 g	α_D^{25} —1.10°	(hom.; *l*, 0.5)
III	90.2°,	30 »	6.1 »	α_D^{25} —2.63°	(hom.; *l*, 1)
IV	90.2°,	30 »	2.65 »	α_D^{25} —2.64°	»
V	90.2°,	30 »	3.1 »	α_D^{25} —2.64°	»
VI	90.2–91°,	30—0.6 mm	1.6 »		
VII	91°,	0.6 mm	3.1 »	α_D^{26} —10.31°	(hom.; *l*, 1)
VIII	91°,	0.6 »	5.26 »	α_D^{26} —10.54°	»
				α_D^{21} —10.67°	»

Fractions III, IV, and V consist of D(−)-*methyl 3-methylheptanoate* (11.85 g or 30 % of the theoretical). d^{25} 0.871.

Optical rotation α_D^{25} —2.64° (homogeneous; *l*, 1)
$[\alpha]_D^{25}$ —3.30° $[M]_D^{25}$ —4.80°

Anal.: Calcd for $C_9H_{18}O_2$ (158.2) C, 68.31; H, 11.47 %
Found C, 68.34; H, 11.60 %

L(−)-*3-Methylheptanoic acid.* On hydrolysis 11 g of the methyl ester just described gave 9.67 g (96 % of the theoretical) of L(−)-3-methylheptanoic acid, b. p. 80°, 1 mm. d_4^{21} 0.907.

Optical rotation α_D^{21} —3.63° (homogeneous; *l*, 1)
$[\alpha]_D^{21}$ —4.00° $[M]_D^{21}$ —5.77°

L(−)-*3-Methylheptanamide* was prepared via the acid chloride in the usual manner, using 0.42 g of acid initially. After crystallization from benzene-light petroleum (b. p. 40–60°) 0.29 g (70 % of the theoretical) of *amide* was obtained in the form of beautiful silky needles, m. p. 80.8–81.0°. After recrystallization from the same solvent mixture the m. p. was 81.0–81.4°, unchanged after previous melting.

Anal.: Calcd for $C_8H_{17}ON$ (143.2) N, 9.78 %
Found N, 9.59 %

D(−)-*Methyl 3-keto-5-methylheptanoate.* 3.76 g of D(−)-3-methylpentanoic acid was converted into the acid chloride by means of oxalyl chloride, the procedure being that already described in connexion with the preparation of the amide. The acid chloride was allowed to react with methyl sodio-acetoacetate prepared from 0.82 g of sodium and 5.3 g of methyl acetoacetate in benzene (65 ml) solution. The acylation and the subsequent decomposition of the acylation product were carried out in the manner described in detail previously (15). Distillation gave 3.39 g (61 % of the theoretical) of colourless D(−)-*methyl 3-keto-5-methylheptanoate* b. p. 115–117°, 25 mm. d_4^{23} 0.972.

Optical rotation $\alpha_D^{23} -5.65^\circ$ (homogeneous; *l*, 1)

The optical rotation of the β-keto ester will vary with the enol content.

Anal.: Calcd for $C_9H_{16}O_3$	(172.2)	C, 62.76;	H, 9.37 %
Found		C, 62.59;	H, 9.35 %

D(+)-*Methyl 3-keto-5-methyloctanoate* (VIII). 8.0 g of D(+)-3-methylhexanoic acid gave, via the standard procedure, 6.36 g (56 % of the theoretical) of colourless D(+)-*methyl 3-keto-5-methyloctanoate*, b. p. 68–71°, 1 mm. d_4^{23} 0.956.

Optical rotation $\alpha_D^{23} +0.46^\circ$ (homogeneous; *l*, 0.5)

Anal.: Calcd for $C_{10}H_{18}O_3$	(186.2)	C, 64.49;	H, 9.74 %
Found		C, 64.18;	H, 9.84 %

L(−)-*Methyl 3-keto-5-methylnonanoate.* On distillation of the crude product from the β-keto ester preparation starting from L(−)-3-methylheptanoic acid (8.58 g) two colourless fractions, b. p. 73–76°, 1 mm (2.76 g), and 76–79°, 1.0–1.2 mm (5.55 g) respectively, were collected. These were found to have identical density and optical rotation, and the yield of L(−)-*methyl 3-keto-5-methylnonanoate* was therefore 8.31 g or 70 % of the theoretical. d_4^{23} 0.948.

Optical rotation $\alpha_D^{23} -1.92^\circ$ (homogeneous; *l*, 1)

Anal.: Calcd for $C_{11}H_{20}O_2$	(200.3)	C, 65.97;	H, 10.07 %
Found		C, 65.67;	H, 10.07 %

D(−)-*19-keto-21-methyltricosanoic acid.* D-Methyl 3-keto-5-methylheptanoate (2.78 g), methyl 17-iodoheptadecanoate (6.64 g, the iodo-ester, m. p. 52.2–52.9°, was prepared by the method of HUNSDIECKER and HUNSDIECKER (23)); dry potassium carbonate and dry methyl-*n*-propyl ketone (25 ml) were boiled under reflux for 18 hours. The reaction mixture was worked up, and hydrolysis and ketonic cleavage carried out following the standard procedure (cf. *e.g.* Part I (16)). Crystallization from acetone gave 4.05 g (65 % of the theoretical) of D(−)-*19-keto-21-methyltricosanoic acid* in the form of elongated lustrous plates. M. p. 75.4–76.4°. Recrystallization from acetone raised the m. p. to 76.9–77.1°. After previous melting the m. p. was unchanged. Long X-ray spacing (pressed or melted specimen) 42.6 Å.

Optical rotation $\alpha_D^{22} -0.68^\circ$ (chloroform; *l*, 4; *c*, 8.59)
$[\alpha]_D^{22} -1.96^\circ$ $[M]_D^{22} -7.5^\circ$

Anal.: Calcd for $C_{24}H_{46}O_3$	(382.6)	C, 75.34;	H, 12.12 %
Found		C, 75.22;	H, 12.10 %

D(+)-*19-keto-21-methyltetracosanoic acid* (X). The keto-acid was prepared through the usual procedure from D-methyl 3-keto-5-methyloctanoate (VIII) (4.90 g) and methyl 17-iodoheptadecanoate (IX) (10.75 g). Crystallization from acetone gave 6.26 g (63 % of the theoretical) of D(+)-*19-keto-21-methyltetracosanoic acid*, m. p.

70.5–71.0°. After recrystallization from ethanol the keto-acid formed small lustrous plates melting at 70.7–71.3°. The acid solidified in the form of beautiful prismatic plates. Remelting took place at 71.2–71.3°. Long X-ray spacing (pressed or melted specimen) 39.7 Å.

Optical rotation $\alpha_D^{22} + 0.55°$ (chloroform; *l*, 4; *c*, 9.29)
$[\alpha]_D^{22} + 1.48°$ $[M]_D^{22} + 5.7°$

Anal.: Calcd for $C_{25}H_{48}O_3$ (396.6) C, 75.70; H, 12.20 %
Found C, 75.79; H, 12.10 %

L(–)-*19-Keto-21-methylpentacosanoic acid* was prepared from L-methyl 3-keto-5-methylnonanoate (5.0 g) and methyl 17-iodo-heptadecanoate (10.2 g). After hydrolysis and ketonic cleavage the keto-acid was isolated and crystallized from acetone, giving 6.9 g (68 % of the theoretical) in the form of small spherulitic aggregates. M. p. 64.2–64.9°. After previous melting the m. p. was 64.0–64.3°. Recrystallization was attempted from a number of solvents, but the keto-acid in every case separated in the form of a micro-crystalline white powder. After crystallization from light petroleum (b. p. 40–60°), most of the specimen melted at 64.3–65.2°, but a small amount of material did not melt until 66.7°. After previous melting the sample remelted at 64.2–64.6°; on cooling solidification occurred at 64.4°, and on immediate re-heating melting occurred at 66.3–66.7°. The keto-acid is thus dimorphic, but at room temperature both pressed and melted specimens gave X-ray diffraction patterns showing the presence of one form only, with a long spacing of 49.7 Å.

Optical rotation $\alpha_D^{21} - 0.29°$ (chloroform; *l*, 2; *c*, 9.07)
$[\alpha]_D^{21} - 1.60°$ $[M]_D^{21} - 6.6°$

Anal.: Calcd for $C_{26}H_{50}O_3$ (410.7) C, 76.04; H, 12.27 %
Found C, 75.89; H, 12.20 %

D(–)-*21-Methyltricosanoic acid.* The reduction of the C_{24} keto-acid was carried out by CLEMMENSEN's method in ethanol solution, using 3.0 g of keto-acid, 150 g of amalgamated mossy zinc (BAKER's Analyzed), and 200 ml of 99.5 % ethanol. The solution was saturated with dry hydrogen chloride, and boiled under reflux for a total time of 18 hours. Hydrogen chloride was led in at 6 hours intervals. The reaction mixture was worked up and subjected to hydrolysis in the usual manner (cf. *e.g.* Part III). Crystallization from light petroleum (b. p. 40–60°) gave 2.45 g (85 % of the theoretical) of D(–)-*21-methyltricosanoic acid* in the form of beautiful lustrous plates, m. p. 67.2–67.6°. 2.30 G were recrystallized from acetone, giving 1.97 g of m. p. 67.8–68.1°, unchanged after previous melting. Long X-ray spacing (pressed or melted specimen) 39.1 Å.

Optical rotation $\alpha_D^{22} - 0.68°$ (chloroform; *l*, 2; *c*, 9.93)
$[\alpha]_D^{22} - 3.42°$ $[M]_D^{22} - 12.6°$
$\alpha_D^{22} - 0.72°$ (chloroform; *l*, 4; *c*, 5.11)
$[\alpha]_D^{22} - 3.52°$ $[M]_D^{22} - 13.0°$

Anal.: Calcd for $C_{24}H_{48}O_2$ (368.6) C, 78.19; H, 13.13 %
Found C, 78.05; H, 13.16 %

D(–)-*21-Methyltricosanamide* was prepared in the usual manner via the acid chloride. After crystallization from light petroleum (b. p. 95–100°) the *amide* melted at 97.6–97.9°, unchanged after previous melting or after further recrystallization from benzene. The amide had a white, microcrystalline appearance. Long X-ray spacing (pressed specimen) 35.5 Å.

Anal.:	Calcd for $C_{24}H_{49}ON$	(367.6)	N, 3.81 %
	Found		N, 3.84 %

D(−)-*21-Methyltetracosanoic acid* (XI). The reduction of 4.40 g of the keto-acid (VIII) was carried out in the manner described for the C_{24} acid above. Crystallization from light petroleum gave 3.22 g (76 % of the theoretical) of D(−)-*21-methyltetracosanoic acid*, m. p. 59.8–60.2°. Further crystallization from glacial acetic acid gave lustrous plates of m. p. 60.3–60.8°. After previous melting the m. p. was 60.6–60.8°. Long X-ray spacing (pressed or melted specimen) 40.3 Å.

Optical rotation $\alpha_D^{21} - 0.25°$ (chloroform; *l*, 4; *c*, 7.28)
$[\alpha]_D^{21} - 0.86°$ $[M]_D^{21} - 3.3°$

Anal.:	Calcd for $C_{25}H_{50}O_2$	(382.6)	C, 78.47; H, 13.17 %
	Found		C, 78.55; H, 13.22 %

D(−)-*21-Methyltetracosanamide.* After two crystallizations from benzene the microcrystalline *amide* melted at 92.8–93.0°. Crystallization from light petroleum (b. p. 80–100°) gave the amide in the form of small lustrous plates, with the m. p. just recorded. Long X-ray spacing (pressed specimen) 45.5 Å.

Anal.:	Calcd for $C_{25}H_{51}ON$	(381.6)	N, 3.67 %
	Found		N, 3.70 %

L(+)-*21-Methylpentacosanoic acid.* CLEMMENSEN reduction of 4.03 g of L(−)-19-keto-21-methylpentacosanoic acid was performed in ethanol solution with the usual procedure. After hydrolysis of the ester formed during the reduction and crystallization of the free acid from glacial acetic acid, 2.77 g (71 % of the theoretical) of L(+)-*21-methylpentacosanoic acid* were obtained in the form of small lustrous plates, m. p. 55.9–56.2°. Previously melted material had m. p. 53.2–53.5°, indicating dimorphism. After further crystallization from acetone the melting points of the two forms rose to 56.5–56.7° and 54.1° respectively. The lower melting form is stable only near the m. p. The long X-ray spacings of the higher and lower melting forms were found to be 37.7 and 49.2 Å respectively.

Optical rotation $\alpha_D^{24} + 0.14°$ (chloroform; *l*, 4; *c*, 5.25)
$[\alpha]_D^{24} + 0.67°$ $[M]_D^{24} + 2.6°$

Anal.:	Calcd for $C_{26}H_{52}O_2$	(396.6)	C, 78.72; H, 13.21 %
	Found		C, 78.54; H, 13.13 %

L(+)-*21-Methylpentacosanamide* melted, after two crystallizations from light petroleum (b. p. 95–100°), at 93.9–94.2°. Previously melted material had m. p. 93.2°, indicating that the amide is dimorphous. The amide was obtained from light petroleum in microcrystalline form. Long X-ray spacing (pressed specimen) 48.1 Å.

Anal.:	Calcd for $C_{26}H_{53}ON$	(395.6)	N, 3.54 %
	Found		N, 3.60 %

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SUMMARY

Electrolysis of the sodium salts of the dextro- and laevorotatory enantiomorphs of methyl hydrogen β-methylglutarate in methanol solution gives the dextro- and laevorotatory methyl esters respectively of 3,6-dimethylsuberic acid in yields of about 70 % of the theoretical. No racemization is found to occur. Dextro-, laevo-, DL- and *meso*-3,6-dimethylsuberic acid have been prepared. The methyl esters of the optically active forms of the lower β-methyl substituted monocarboxylic acids may conveniently be obtained by mixed electrolysis of the appropriate enantiomorph of methyl hydrogen β-methylglutarate and normal chain acid, the yields being 26–32 % of the theoretical, calculated on the active methyl hydrogen β-methylglutarate used. D(−)-3-Methylpentanoic acid, D(+)- and L(−)-3-methylhexanoic acids, L(−)-3-methylheptanoic acid, and D(+)-3-methylundecanoic acid have been prepared by this route.

D-Methyl 3-keto-5-methylheptanoate, D-methyl 3-keto-5-methyloctanoate, and L-methyl 3-keto-5-methylnonanoate have been synthesized from the appropriate acids. Alkylation of the optically active β-keto esters by methyl 17-iodoheptadecanoate, followed by hydrolysis and ketonic cleavage of the alkylation products, gave D(−)-19-keto-21-methyltricosanoic acid, D(+)-19-keto-21-methyltetracosanoic acid, and L(−)-19-keto-21-methylpentacosanoic acid respectively. Clemmensen reduction in ethanol solution gives the corresponding reduced acids, *i.e.* D(−)-21-methyltricosanoic acid, D(−)-21-methyltetracosanoic acid and L(+)-21-methylpentacosanoic acid respectively.

Institute of Medical Chemistry, University of Uppsala.

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Tryckt den 27 mars 1950

Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 3

Read 7 December 1949

Some homologues of tetraethenyl hexasulphide

By Arne Fredga and Hermann Bauer

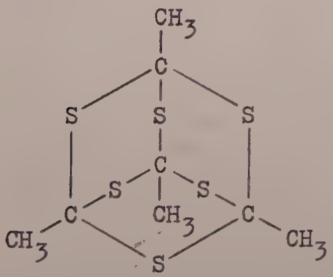
With 1 figure in the text

In an earlier paper (1) a new structural formula for the tetraethenyl hexasulphide (I) was proposed. It contains a very symmetric, strainless system of four six-membered rings, analogous to the well-known ring systems of hexamethylene tetramine and adamantane. It was also pointed out that it is difficult to produce conclusive evidence for the formula by purely chemical methods. The ring system is remarkably stable, but if it is attacked by chemical agents, extensive degradation takes place. If homologous compounds of the general formula II ($R = C_2H_5$, C_3H_7 etc.) are examined, it may be easier to isolate characteristic degradation products. In this way additional proof for the correctness of the new structure might be obtained.

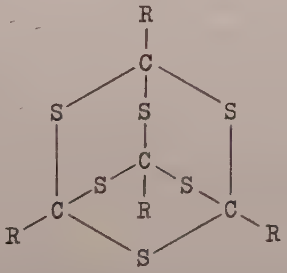
The physico-chemical properties of such homologues, especially those with long side chains, may also be of interest.

The tetraethenyl hexasulphide, $(CH_3C)_4S_6$, can be obtained by treating thiol acetic acid with condensing agents *e.g.* zinc chloride (2, 3) or hydrogen halides (4). A second, rather convenient method was found by Borgeson and Wilkinson (5), who, however, failed to recognize the compound prepared by them as tetraethenyl hexasulphide. They treated acetyl chloride or acetyl bromide with liquid hydrogen sulphide in sealed tubes and after some weeks obtained fine crystals melting at 225° and having the empirical formula $C_4H_6S_3$. On cryoscopic determination in benzene they found the molecular weight 270 instead of 150 corresponding to the formula mentioned above. Nevertheless they proposed the structure III and remarked that the compound is "somewhat associated in benzene solution".

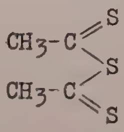
This formula is, however, hardly consistent with the high melting point of the compound. On repeating their experiments, it was found that the product is identical



(I)



(II)



(III)

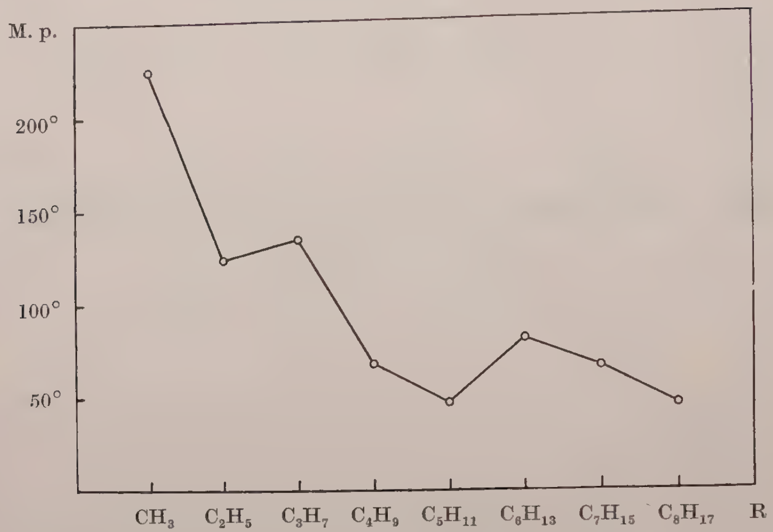


Fig. 1. Melting points of the compounds $(RC)_4S_6$.

with the tetraethenyl hexasulphide previously described by several authors (3, 4). The molecular wight of this compound has been determined by the freezing point method in benzene (3) and naphtalene (4); in both cases values near 300 were found. On determination by the boiling point method in three solvents of different character (benzene, acetone, chloroform), we found in all cases the same value. As this value is found at different temperatures and in different media, there cannot be any doubt that the correct formula is $C_8H_{12}S_6$ and the structure III can be disregarded. The same, of course, applies to another formula corresponding to the lower molecular weight and proposed by Bongartz (2). It has not been seriously discussed by later authors.

The homologues containing *unbranched* side chains can be obtained either through the corresponding thiol acids or directly from acyl chlorides and hydrogen sulphide. The whole series of compounds up to $R = C_8H_{17}$ has been prepared. The compounds crystallise readily and the variation of the melting point within the series is given in figure 1. The yields were not very good, considerable amounts of oily by-products being formed, but a systematic examination of the reactions may possibly lead to improvements resulting in a better yield. The direct method of Borgeson and Wilkinson (5) is more convenient and it seems to give a better yield (calculated on the basis of carboxylic acid). This method will therefore be given preference in the future work.

Experiments with thiol acids cotaining *branched* chains (thiol *iso*-valeric and thiol pivalic acid) have not yielded any crystallised products. It cannot as yet be decided wether the branching of the chain prevents or retards the reaction or wether the condensation products are really formed but have low melting points and fail to crystallise. Bongartz was unable to obtain any crystallised products from thiol benzoic acid (2).

Several of the thiol acids prepared in the course of this investigation have to our knowledge not been described previously. They were prepared by known methods and there was no difficulty in obtaining constant-boiling fractions of the acids giving acceptable values for the equivalent weights. Preliminary titrations with iodine (thiol group) and sulphur determinations tend, however, to give too low values (up to 10 %). The data given for these acids are thus to be regarded as preliminary.

Experimental

Thiol acids.

The thiol acids were obtained in most cases by the method given by SUNNER and NILSSON (6). It was developed especially for thiol acetic acid, but is equally applicable to the homologues. At least for the higher acids, it is far superior to the old method of KEKULÉ (distillation of carboxylic acid with phosphorus pentasulphide).

A rapid stream of hydrogen sulphide is passed through 200 ml of dry pyridine with effective cooling and vigorous stirring, and 40–50 g of acyl chloride are added carefully drop by drop. Finally the necessary amount of 5-*N* sulphuric acid is added, the organic acid is taken up in ether and dried with calcium chloride or sodium sulphate. After evaporation of the ether, the product is rectified in a nitrogen atmosphere (the higher homologues at reduced pressure) until a constant boiling point is obtained. Further particulars concerning the method are found in the paper of SUNNER and NILSSON (6).

Thiol propionic acid. This acid has been prepared by KITAMURA using the KEKULÉ method (7). We prepared it by both methods and obtained identical products. 40 g propionyl chloride yielded 15 g of pure acid with b. p. 108–109°.

Thiol n-*butyric acid.* After two distillations the b. p. was 119–120°. 40 g of butyryl chloride yielded 16 g acid.

0.2236 g acid: 21.30 ml 0.0997-*N* NaOH.

C_4H_8OS Equiv.wt calc. 104.2 found 105.3

Thiol n-*valeric acid.* After three distillations the b. p. was 138–140°.

0.1369 g acid: 11.75 ml 0.0997-*N* NaOH

$C_5H_{10}OS$ Equiv.wt calc. 118.2 found 116.9

Thiol iso-*valeric acid.* After 6 distillations the b. p. was 121° (sharp). 45 g of *iso*-valeryl chloride yielded 13 g acid.

0.3684 g acid: 31.39 ml 0.0998-*N* NaOH.

$C_5H_{10}OS$ Equiv.wt. calc. 118.2 found 117.6

Thiol pivalic acid. After 2 distillations the b. p. was 125–127° 45 g of pivaloyl chloride yielded 22 g acid.

0.2888 g acid: 24.45 ml 0.0998-*N* NaOH.

$C_5H_{10}OS$ Equiv.wt. calc. 118.2 found 118.4

Thiol n-*capronic acid.* After 3 distillations the b. p. was 98–101° (65 mm Hg).

0.2360 g acid: 17.30 ml 0.1024-*N* NaOH.

$C_6H_{12}OS$ Equiv.wt. calc. 132.2 found 133.2

Thiol n-*heptanoic acid.* After 3 destillations the b. p. was 81° (11 mm Hg). 50 g of heptoyl chloride yielded 11 g acid.

0.3060 g acid: 20.00 ml 0.0997-*N* NaOH.

$C_7H_{14}OS$ Equiv.wt calc. 146.2 found 153.5

Thiol n-*octanoic acid.* After 3 distillations the b. p. was 95° (10 mm Hg).

0.3376 g acid: 21.35 ml 0.0966-*N* NaOH.

$C_8H_{16}OS$ Equiv.wt. calc. 160.3 found 163.3

Thiol n-*nonanoic acid.* After 3 distillations the b. p. was 105° (10 mm Hg).

0.5089 g acid: 29.45 ml 0.0957-*N* NaOH.

$C_9H_{18}OS$ Equiv.wt. calc. 174.3 found 180.6

Determination of the molecular weight of tetraethenyl hexasulphide ($C_8H_{12}S_6 = 300.6$).

0.1257, 0.2215, 0.3102 g in 15.0 g *benzene* (k = 2.67): $\triangle = 0.075°$, 0.137°, 0.195°. Mol.wt. found 298, 288, 283

0.1690, 0.3040, 0.4125, 0.4933 g in 15.4 g *acetone* (k = 1.67): $\triangle = 0.060°$, 0.106°, 0.144°, 0.171°. Mol. wt. found 305, 311, 311, 313.

0.2481, 0.3489, 0.4317 g in 18.2 g *chloroform* (k = 3.66): $\triangle = 0.161°$, 0.236°, 0.292°. Mol. wt. found 310, 297, 297.

Homologues of tetraethenyl hexasulphide.

Preparation through thiol acids. Three parts of the thiol acid were mixed with two parts of water-free zinc chloride and left to stand for several days. The mixture must be protected against moisture and the zinc chloride should be freshly melted and coarsely powdered. The optimal temperature seems to be room temperature or (for the higher homologues) 30–40°. After some time hydrogen sulphide is evolved, and crystals are formed, and at length the mixture solidifies more or less completely. When the reaction has come to an end and no more crystals are formed, 2-*N* hydrochloric acid is added to dissolve the zinc compounds. The organic material is taken up in benzene, the solution is washed several times with sodium carbonate solution and evaporated to dryness. The reddish-brown, crystalline but more or less smeary residue is recrystallised several times from ethanol or propanol, if necessary with charcoal. In some cases it was found convenient to decolourise the product by treatment with charcoal in cold acetone solution. The compounds are sparingly soluble in methanol and ethanol, more soluble in higher alcohols and easily so in ether, benzene and ethyl acetate.

The condensation can also be performed with hydrogen chloride, but in this case the reaction proceeds rather slowly. Water-free aluminium chloride gives rapid condensation, but neither the yield nor the quality of the product is satisfactory.

Preparation from acyl chlorides. The reaction is effected in sealed tubes and it was found convenient to add a certain amount of water-free zinc chloride as condensing agent. 10 ml of acid chloride and 5 g of zinc chloride are brought into the tube and the whole is chilled in a mixture of ethanol and solid carbon dioxide. A rapid current of hydrogen sulphide is passed into the tube until 10–15 ml have been condensed. The tube is then sealed and left to stand at room temperature for about one week. It is then chilled again and opened, the hydrogen sulphide is evaporated and the residue is worked up as described above.

The following compounds were prepared from the corresponding thiol acids. Most of them were also prepared directly from the acyl chlorides. They were all obtained as quite colourless crystals; on slow evaporation of a solution in a suitable solvent rather large crystals are formed.

$(C_2H_5C)_4S_6$. 12 g thiol propionic acid gave 1.1 g pure hexasulphide forming short-prismatic crystals or double pyramids. M. p. 122.5–123.5°.

39.13 mg sbst.: 57.51 mg CO_2 and 19.47 mg H_2O. 17.51 mg sbst.: 68.48 mg $BaSO_4$.
$C_{12}H_{20}S_6$ (356.7) calc. C 40.41 H 5.65 S 53.94
found C 40.11 H 5.57 S 53.72
0.1276, 0.3068 g sbst. in 15.15 g benzene: $\triangle = 0.081°$, $0.148°$.
Mol wt. calc. 356.7 found 375, 365.

$(C_3H_7C)_4S_6$. 14 g thiol butyric acid yielded 2.3 g pure hexasulphide forming rectangular plates or flakes. M. p. 133.5–134°.

26.75 mg sbst.: 45.28 mg CO_2 and 16.16 mg H_2O. 19.54 mg sbst.: 5.728 ml 0.0997-*N* NaOH.
$C_{16}H_{28}S_6$ (412.7) calc. C 46.56 H 6.84 S 46.62
found C 46.19 H 6.76 S 46.86
0.1802, 0.3066, 0.4300 g sbst. in 16.0 ml benzene: $\triangle = 0.070°$, $0.123°$, $0.176°$.
Mol wt. calc. 412.7 found 429, 416, 408.

$(C_4H_9C)_4S_6$. The compound forms fine, glistening needles having m. p. 66–67°.

17.90 mg sbst.: 33.50 mg CO_2 and 12.19 mg H_2O. 21.54 mg sbst.: 7.432 ml 0.0738-*N* NaOH.
$C_{20}H_{36}S_6$ (468.9) calc. C 51.23 H 7.74 S 41.03
found C 51.07 H 7.62 S 40.82
0.1202, 0.2420 g sbst. in 13.9 g benzene: $\triangle = 0.048°$, $0.105°$.
Mol. wt. calc. 468.9 found 480, 440.

$(C_5H_{11}C)_4S_6$. The compound was obtained as diamond-shaped plates or flakes. The following homologues all have a similar crystal habit. M. p. 45.5–46.5°.

16.47 mg sbst.: 33.10 mg CO_2 and 12.38 mg H_2O. 20.30 mg sbst.: 6.270 ml 0.0738-*N* NaOH.
$C_{24}H_{44}S_6$ (525.0) calc. C 54.90 H 8.44 S 36:65
found C 54.84 H 8.41 S 36.54

$(C_6H_{13}C)_4S_6$. The compound was obtained as diamond-shaped plates or flakes. M. p. 79.5–80.5°.

19.27 mg sbst.: 40.99 mg CO_2 and 15.48 mg H_2O. 24.27 mg sbst.: 5.043 ml 0.0997-*N* NaOH.
$C_{28}H_{52}S_6$ (581.1) calc. C 57.87 H 9.02 S 33.11
found C 58.05 H 8.99 S 33.21
0.1676 g sbst. in 15.35 ml benzene: $\triangle = 0.050°$.
Mol. wt. calc. 581.1 found 580.

$(C_7H_{15}C)_4S_6$. The compound was obtained as diamond-shaped plates or flakes M. p. 65–65.5°.

19.26 mg sbst.: 42.30 mg CO_2 and 15.72 mg H_2O.
$C_{32}H_{60}S$ (637.2) calc. C 60.32 H 9.49
found C 59.93 H 9.14

$(C_8H_{17}C)_4S_6$. The compound was obtained as diamond-shaped plates or flakes. M. p. 44–45°.

17.86 mg sbst.: 40.56 mg CO_2 and 15.74 mg H_2O.
$C_{36}H_{68}S_6$ (693.3) calc. C 62.36 H 9.89
found C 61.97 H 9.86

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SUMMARY

Seven homologues of tetraethenyl hexasulphide having unbranched side chains have been prepared (see formula II p. 113; $R = C_2H_5$, C_3H_7,. . . . . C_8H_{17}). It is pointed out that a compound $C_4H_6S_3$ described in the literature as "the anhydride of dithioacetic acid" (5) has the double molecular weight and is identical with tetraethenyl hexasulphide.

The investigation is continued.

Department of Organic Chemistry, Chemical Institute, University of Uppsala, December 1949.

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Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 4

Mitgeteilt am 7. Dezember 1949

Über spontane Regression implantierter Tumorgewebe

Von Beth von Euler und Hans von Euler

Bei den sehr zahlreichen Untersuchungen, welche hier und von anderen Arbeitskreisen über die tumorhemmenden Wirkungen cancerogener, anti-cancerogener und anti-mitotischer Stoffe ausgeführt wurden, ist die Auswertung der Versuchsresultate durch das häufige Auftreten spontaner Regressionen sehr erschwert worden.[1] Diese Schwierigkeiten haben öfters veranlasst, die Bedingungen, unter welchen Versuchstiere sich gegen die Entwicklung von Tumoren resistent erweisen, sowie die Bedingungen, unter welchen eine solche Resistenz von ,,virulenten" Geweben oder Zellen überwunden werden kann, zu studieren.

Das hier auftretende Problem der ,,Abwehrkräfte" von Individuen ist zweifellos eines der wichtigsten sowohl in der Therapie wie in der Biochemie der Tumoren. Wir haben in der letzten Zeit mehrfach Gelegenheit gehabt anlässlich von Studien über die Freund-Kaminer'sche Reaktion[2] demselben nahezutreten, da ja Freund und Kaminer von der Grundidee ausgingen, dass die Abwehr, welche der normale Organismus gegen Tumorzellen durch Auflösung derselben zu leisten vermag, bei tumorkranken Individuen geschädigt ist.

Die Abwehrfähigkeit eines Individuums gegen implantiertes Tumorgewebe oder gegen ev. eingeführte Tumorzellen (oder Extrakte derselben) hat man gewöhnlich als ,,Resistenz" bezeichnet. Man hat aber dabei zwei Teilvorgänge oft nicht hinreichend scharf unterschieden[3]: nämlich einerseits das Einwachsen eines Implantates in das Wirtsgewebe und andererseits die Weiterentwicklung desselben im Wirtsorganismus. Die vielen einander widersprechenden Angaben der Literatur sind zum grossen Teil auf den Mangel einer einheitlichen und klaren Nomenklatur zurückzuführen. Wir werden also in der vorliegenden und den folgenden Mitteilungen die nachstehenden Bezeichnungen verwenden.

,,Angegangene Implantate" und ,,stetig wachsende Implantate"

Von der näheren Besprechung der ,,angegangenen Implantate", also des Einwachsens des Implantates in das Wirtsgewebe wollen wir hier zunächst absehen, da dieser Vorgang von den geweblichen Eigenschaften, wie z. B. der Härte, der

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[1] Euler, B. Skarzynski u. Rönnestam-Säberg, Dieses Arkiv 17 A 24 (1943). — Euler, Ahlström u. B. Skarzynski, ebenda 17 A No 29 (1943). — Euler, H. Hasselquist u. U. Lööv, Dieses Arkiv 25 A No 14 (1948). — Euler u. W. Solodkowska, Dieses Arkiv 24 A No 24 (1947).

[2] Euler, L. Heller u. Chr. Curman, Dieses Arkiv 1, No 8 (1948) und 1, No 36 (1949). — Euler u. L. Heller, Dieses Arkiv 1, No 49 (1949).

[3] Vgl. Euler u. B. Skarzynski, Biochemie der Tumoren, Stuttgart 1941, S. 189–200.

Struktur und dem Wassergehalt des Tumors stark beeinflusst wird; dieser Vorgang muss histologisch verfolgt und charakterisiert werden.

Wir wollen in dieser Mitteilung vielmehr die *Entwicklung* des eingewachsenen Implantates betrachten.

Eine strenge, auf mikroskopische Beobachtungen begründete Scheidung zwischen „Angehen“ und „Entwicklung“ des implantierten Tumors ist allerdings erst einige Zeit nach der Implantation durchführbar, da Wachstum und Regression des Implantates von der vorhergehenden Infiltration bezw. Abkapselung des Implantates, somit von dessen Stoffaustausch mit dem Wirtsgewebe abhängig sind.

Die Entwicklung angegangener Implantate ist jedenfalls derjenige Vorgang, an welchem sich die Immunität des Individuums, andererseits die Virulenz des Implantates verhältnismässig rein geltend macht. Diese Entwicklung haben wir meist an subcutanen, zuweilen an intramuskularen Sarkomimplantaten untersucht. Zur Beurteilung unserer Ergebnisse über den Einfluss von cancerogenen, anti-cancerogenen und anti-mitotischen Stoffen auf das Schicksal des Tumors war es erforderlich, Erfahrungen an nicht injicierten Tumor-Ratten zu sammeln.

Experimentelle Angaben

Unser aus Norwegen (Dr. OLAV SOPP) eingeführter reiner Stamm weisser Ratten wurde seit 1927 in unserem Vitamin-Institut weiter gezüchtet. Die Jensensarkome, die auf Sarkomratten zurückgehen, welche wir Herrn Prof. Dr. C. KREBS, Aarhus, verdanken, wurden 1935 auf unseren Rattenstamm übertragen und auf diesem seither durch viele Passagen weiter gezüchtet.

Die zu Sarkom-Versuchen verwendeten Ratten erhielten gemischte natürliche Kost, bestehend hauptsächlich aus Mais, Kleie, Milch und Hefe mit Zusätzen von Gemüse, Brot und Fisch oder Leber.

Die Tiere wurden in den 8 kälteren Monaten bei etwa 25° gehalten. Jahreszeitliche Schwankungen im physiologischen Zustand der Tiere machten sich bemerkbar.

Impfung in der Regel mit 20 mg Sarkomschnitt.

Spontane Regressionen implantierter Sarkome

Einleitend sei über „angegangene Implantate“ und „stetig wachsende Implantate“ bei früheren Versuchsserien des Institutes folgendes mitgeteilt:

Jensen-Sarkome

Aus den Jahren 1935–1939 besitzen wir keine vollständigen Tabellen über die procentische Anzahl angegangener Implantate von Jensen-Sarkomen. Es kann aber gesagt werden, dass diese Zahl in den letzten Jahren abgenommen hat, während die procentische Zahl der Regressionen nunmehr bedeutend grösser ist als früher.

Einem Material von 234 Fällen aus dem Jahr 1949 entnehmen wir die per 100 Ratten berechneten, abgerundeten Mittelwerte der Implantate:

Nicht angegangen	Angegangen
25	75

Stetig wachsend	Retrograd
24	51

An einer erheblich kleineren Anzahl unserer Ratten mit einem aus Benzpyren entwickelten Tumor (eingeimpfte Menge je 20 mg) wurde gefunden

Nicht angegangen	Stetig wachsendes Impl.	Retrogrades Impl.
50 %	20 %	30 %

Das Ergebnis, dass mit einem Benzpyren-Sarkom eine viel schlechtere Ausbeute an Tumoren erhalten wurde als mit Jensen-Sarkomen wurde darauf zurückgeführt, dass das Jensen-Sarkom schon in vielen Generationen an unserem Rattenstam übergeimpft worden war.

Unsere neueren Erfahrungen an 204 Tieren mit Benzpyren-Tumoren, welche in diesem Jahr (1949) durch Einführen einer Benzpyren-Arachidol-Emulsion erhalten worden waren und seit dieser Zeit etwa 8—16 Passagen durchgemacht hatten, sind folgende:

Nicht angegangen	Angegangen	
24 %	76 %	
	Stetig wachsend	Retrograd
	35 %	41 %

Wie wir schon früher fanden, zeigten in den letzten Jahren die Benzpyren-Sarkome, und zwar nicht nur die direkt durch Injicierung von Benzpyren hervorgerufenen sondern auch die durch Transplantierungen daraus gewonnenen eine durchweg kompaktere und härtere Konsistenz als die Jensen-Sarkome.

Mit *Methylcholanthren-Sarkomen* wurden in den Jahren 1942—43 bei Implantationen (I. RÖNNESTAM-SÄBERG) nur sehr wenig (5—10 %) „Angänge" erzielt.

Auf die zwar nicht absolute, aber relativ hohe Specifität der chemisch inducierten Impftumoren haben wir unter Hinweis auf Ergebnisse anderer Forscher (LEWIS u. LICHTENSTEIN u. a.) schon früher (Dieses Arkiv 17 Nr. 24) aufmerksam gemacht.

Die Zahl der Regressionen ist durch verschiedene Umstände bedingt; die Immunität des Wirtsorganismus spielt natürlich eine wesentliche Rolle; aber auch die Art der Impfung und die Vorgeschichte, ganz besonders die Virulenz, des implantierten Tumors beeinflussen das Resultat, ebenso die Grösse und Behandlung des Implantates. UHLENHUTH[1] hat schon das Vorkommen individueller Unterschiede hinsichtlich der natürlichen Immunität gegen Tumoren betont. Andererseits ist bekanntlich die Empfänglichkeit verschiedener Organe der gleichen Tier-Art- und Rasse sehr ungleich. Die leichte Überimpfbarkeit des Jensen-Sarkoms auf Ratten-Gehirn hat sich kürzlich bei Versuchen gezeigt, die F. SOAVE[2] in diesem Institut ausgeführt hat.

Hervorrufung der Resistenz (Immunität) gegen Sarkom-Implantate

Die Hervorrufung von Resistenz gegen Tumor-Implantation durch vorhergehende Impfung der Tiere mit dem gleichen Tumor geht aus zahlreichen Untersuchungen hervor, unter denen diejenigen von DOMAGK und HACKMAN[3], von UHLENHUTH[4], CLEMESEN[5] u. a. genannt seien.

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[1] UHLENHUTH, Arb. a. d. Reichsgesundheitsamt 30, 434 (1909).

[2] F. SOAVE, Dieses Arkiv 1, No 57 (1950).

[3] DOMAGK u. HACKMANN, Z. Krebsforsch. 42, 192 (1935). — DOMAGK, ebenda 56, 247 (1949). — DRUCKREY, ebenda 56, 407 (1949).

[4] P. UHLENHUTH, Z. Immunitätsf. 90, 98 (1937).

[5] CLEMESEN, Diss. Kopenhagen 1938.

Bei negativem Ausfall der 1. Impfung (mit Jensen-Sarkom) kann das betr. Tier mit ziemlicher Sicherheit als resistent bezw. immun gegen den gleichen Tumor betrachtet werden. In einer kleineren, in diesem Jahr angestellten Versuchsserie, war die Zeit zwischen der 1. und der 2. Impfung 1 bezw. 2 Monate. Der Erfolg der 2. Impfung war in sämtlichen Fällen negativ.

Tab. 1

Versuchsserie. 1949

Ratte No.	Geschl.	Geboren	1. Impfung	2. Impfung	Erfolg
109 10	♀			14. Sept.	neg.
12	♀	12.–17. Jan.			»
06	♀		27. Juli		»
09	♀	15.–20. April			»
15	♀				»
109 42	♀				»
49	♀				»
51	♀	12.–17. Jan.	10. August		»
52	♀				»
53	♀				»
54	♀				»

Was die Specifität der natürlichen und der durch Jensensarkom-Implantation verstärkten Resistenz betrifft, so widersprechen sich auch in diesem Punkt die vorliegenden Literatur-Angaben.

EULER, SKARZYNSKI und SÄBERG gaben 1944 Ergebnisse von Versuchen an, bei welchen Ratten, die sich gegen Jensensarkome resistent erwiesen hatten, Injektionen von Benzpyren in Glycerin emulgiert bezw. Methylcholanthren erhielten. In 2 Fällen von 5 erhielten wir nach Benzpyren-Injektion nach 130 bezw. 155 Tagen kräftig wachsende Tumoren. Eine Wiederholung dieses Versuches im folgenden Jahr mit 10 Ratten, welchen 2 Tage die Nahrung entzogen worden war, ergab in 3 Fällen Tumoren. Als aber der Versuch in der Weise abgeändert wurde, dass die gegen Jensensarkom resistenten Ratten Implantationen von Benzpyren-*sarkomen* erhielten, wurde nur in 1 Fall von 10 Angehen des Implantates konstatiert, das nach 14 Tagen retrograd wurde.

Impfversuche mittels zellfreier Extrakte (I. RÖNNESTAM)

Die Möglichkeit der Tumor-Übertragung durch zellfreie Extrakte ist an Hühnersarkomen bekanntlich durch PEYTON ROUS und durch MURPHY gezeigt worden. Angaben über erfolgreiche zellfreie Übertragung von Jensen-Sarkom liegen vor von HAAGEN[2], hinsichtlich Chemotumoren u. a. von PARSONS[3]. Demgegenüber stehen die negativen Resultate, welche von DOMAGK und HACKMANN[4], DITTMAR[5]

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[1] EULER, B. SKARZYNSKI u. SÄBERG, Dieses Arkiv 17 A No 24 (1943).
[2] HAAGEN, Klin. Wochenschr. 1927 II, 1272.
[3] PARSONS, J. of Path. 43, 1 (1936).
[4] G. DOMAGK u. HACKMANN, Z. Krebsf. 42, 192 (1935).
[5] C. DITTMAR, Z. Krebsf. 45, 109 (1937).

und KLINKE[1] in eingehenden Untersuchungen erhalten worden waren, und auch in der neueren Literatur Bestätigung fanden.

Im Anschluss an Arbeiten dieses Institutes über die Isolierung von Zellkernen nach einem etwas modificierten[2] Verfahren von DOUNCE[3], MIRSKY und POLLISTER[4] u. a. durch Gefrieren und Wiederauftauen von Organbreien in Citratlösungen wurde hier versucht, ob nicht die Übertragbarkeit von Jensen-Sarkom durch zellfreie Extrakte durch wirksamere, aber zugleich sehr schonende Methoden der Abtrennung von den Zellen, als sie bei früheren Versuchen zur Anwendung gekommen waren, erreicht werden kann. Wir verweisen bezgl. der Methodik auf die erwähnten Beschreibungen[2, 3, 4] und wollen hier nur hervorheben, dass die Behandlung des auftauenden Materiales bei pH 4 erfolgte. Auch die folgende Behandlung des Extraktes (Filtration durch Ton-Filter) wurde unter Abkühlung vorgenommen.

Die Injektion des Extraktes wurde in normale, 3 Monate alte Ratten intramuskular und subcutan vorgenommen, jedesmal mit 0,1–0,3 ml. Nach 3 Wochen erfolgte eine zweite Injektion mit der gleichen Menge.

Die Tiere wurden 4 Monate lang beobachtet, ohne dass eine Spur von Tumorbildung nachgewiesen werden konnte. Sie erhielten dann eine 3. Injektion und wurden weiter beobachtet.

Nach 4 Monaten wurden 3 männliche Ratten auf den Brenztraubensäuregehalt des Blutes untersucht; und zwar mit der gleichen von B. HÖGBERG und F. SCHLENK modificierten Methodik von LU, welche mehrmals beschrieben wurde.[5]

Während der Brenztraubensäure(BTS)-Gehalt des Blutes unserer Normal-Ratten sich recht konstant bei 12–13 γ BTS/ccm hält, und bei sarkomtragenden Ratten je nach Grösse des Sarkoms auf 18–45 γ/ccm Blut ansteigt, wurden bei den dreimal mit dem Extrakt injicierten Ratten die *deutlich erhöhten Werte* gefunden:

20 γ BTS/ccm Blut 16 γ BTS/ccm Blut und 18 γ BTS/ccm Blut.

In Parallelversuchen, bei welchen normale Ratten eine einmalige Injektion des gleichen Extraktvolumens (0,1 ml) erhielten und nach 14 Stunden zur Untersuchung kamen, ergaben sich die durchaus normalen BTS-Werte 12,5 γ bezw. 13 γ/ccm Blut.

Von den 14 Versuchsratten wurden im Lauf des Versuchs 3 unbrauchbar.

Die übrigen 8 Ratten wurden in der üblichen Weise mit Jensen-Sarkom geimpft, und blieben 14 Wochen unter Beobachtung. Während dieser Periode erlag 1 Ratte einer nicht genau erkannten Infektion und musste getötet werden. *Die übrigen 7 Versuchsratten verhielten sich gegen die Jensen-Sarkom-Implantation sämtlich vollkommen resistent, wie nach der Impfung mit Sarkomgewebe.*

Aus dem noch kleinen Versuchsmaterial können noch keine bindenden Schlüsse gezogen werden. Immerhin betrachten wir die beiden erzielten Effekte der Sarkom-Extrakte, nämlich

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[1] J. KLINKE, Arch. klin. Chirurgie 188 591 (1937). — Siehe auch I. MANN, Brit. med. J. 1949, 251 und W. G. GYE, ebenda 1949, 511.

[2] EULER, I. FISCHER, L. HAHN, H. HASSELQUIST u. M. JAARMA, Dieses Arkiv 21 A Nr. 12 (1945).

[3] DOUNCE, J. Biol. Chem. 147, 685 (1943)–151 221 (1943).

[4] MIRSKY u. POLLISTER, Proc. Nat. Acad. Sci. 28, 344 (1942). — Biol. Symp. 10, 247 (1943).

[5] EULER, L. AHLSTRÖM, I. RÖNNESTAM-SÄBERG u. I. PETTERSSON, Z. Krebsf. 55, 15 (1944).

1) den erhöhten BTS-Gehalt des Blutes,

2) die nach der Injektion des Extraktes auftretende Resistenz bemerkenswert, und *ziehen sie als Wirkungen eines tumoreigenen Wuchsstoffes oder vielleicht schon eines Selbstreproduktions-fähigen* (ev. Virus-artigen) *Extraktstoffes in Betracht.*

Resistenz nach Einwirkung tumorhemmender Substanzen

Die Existenz einer tumorhemmenden Substanz in der Milz und überhaupt im retikulo-endothelialen System kann als sicher nachgewiesen gelten. Unbekannt sind noch die Wirkungsweisen dieses Stoffes bezw. dieser Stoffgruppe und die Bedingungen ihrer Bildung. Vom biochemischen Standpunkt aus ist insbesondere das Verhältnis der mit dem retikuloendothelialen System in direkter Beziehung stehenden Wirkungsstoffe zu den oxydo-reduktiven Enzymsystemen, deren Rolle insofern erkannt ist als das niedrige Oxydationspotential der Tumorgewebe selbst experimentell erwiesen ist.

Wie einleitend erwähnt, ist es bei Tierversuchen mit chemotherapeutischen auf Tumorhemmung zu prüfenden Substanzen besonders schwierig, die natürlichen Abwehrkräfte der Versuchstiere während einer Versuchsperiode konstant zu halten.

Die S. 2 erwähnte sehr grosse Zahl von Regressionen, welche bei unserem mit Jensen-Sarkom geimpften Rattenstam vorkommt, hat uns veranlasst, die Wirkung der Blockierung des retikulo-endothelialen Systems (RES) zu untersuchen.

Bekanntlich sind, um die Ausbildung von Abwehr-Reaktionen zu verhindern, schon zahlreiche kolloide Stoffe zur Anwendung gekommen, am öftesten wohl Trypanblau und chinesische Tusche. An Jensen-Sarkomen geschah dies allerdings nicht mit konstantem Erfolg: Während BARONI[1] über die Hervorrufung von Tumoren durch Tusche berichtet, und auch MEYER die Zahl der Tumorangänge in dieser Weise erhöhen konnte, kamen z. B. KIMURA[2] und ENGEL zu negativen Ergebnissen.

Nach früheren zweifelhaften Resultaten mit Organ-Extrakten[3] und kolloidem Fe haben wir nun — zunächst aus praktischen Gründen — diese Versuche und zwar mit *Cholsäure* und mit *Kobalt-Chelaten* wieder aufgenommen.

Die Cholsäure wurde in Form einer feinen Emulsion unseren Ratten intramuskular eingeführt, um die Wirkung möglichst zu protrahieren.

Dosen bis zu 20 mg wurden von den Tieren gut vertragen. Die Versuche sind noch nicht abgeschlossen, indessen kann bereits gesagt werden, dass eine Abnahme der Regressionen bemerkbar ist.

Die Wirkungen der Kobaltpräparate sind schwerer zu beurteilen, weil sich hier auch Enzymhemmungen[4] geltend machen.

Stockholm, Universität, Vitamininstitut.

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[1] BARONI, C. r. Soc. Biol. (Paris) 111, 1108 (1932),

[2] KIMURA, Trans. jap. Path. Soc. 18, 589 (1929).

[3] EULER, J. SÄBERG u. B. HÖGBERG, Z. physiol. Chem. 268, 235 (1941).

[4] EULER u. A. GLASER, Dtsch. Med. Wochenschr. 75; 1950. Im Druck.

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ARKIV FÖR KEMI Band 2 nr 5

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Some observations on the purification of "malt amylase"

By Oskar Holmbergh

The "malt amylase" is a mixture of two enzymes, α- and β-amylase. They have widely different thermostability but seem to behave almost identically in the ultracentrifuge and in electrophoresis. In fractionation, e.g. with precipitating reagents, the two enzymes seem to follow each other closely.[1] In the present investigation no attempt was made to separate the two enzymes; the only purpose was the preparation of highly purified "malt amylase" with as little loss of activity as possible.

The enzymes are much less sensitive to a number of purification processes — mainly the precipitation of impurities consisting chiefly of proteins — if, after extraction of the raw material with water in the proportion 1 : 45 for an hour and centrifuging, the extract is evaporated to about 1/10 of its volume. It seems that the mixture of starch degradation products in the extract protects the enzymes.

Large quantities of proteins can be precipitated from such concentrated, ice-cold extracts without any loss of enzyme activity by dissolving finely powdered solid barium hydroxide, $Ba(OH)_2 + 8\,H_2O$, and stirring vigorously. The barium hydroxide is added until the solution turns thymolphthalein definitely blue (the pH is then about 9.5). The advantage of using solid barium hydroxide is obvious since large amounts of barium hydroxide solution would otherwise be required.

The precipitation of the inert protein — which may to some extent occur in the solution in combination with carbohydrate — is aided by increasing the temperature of the solution, an increase which seems to decrease the combination of protein with carbohydrate. It is interesting that if the precipitate is allowed to settle, for instance over night, but is shaken up before centrifuging, it is impossible to centrifuge the solution clear. This might be due to the combination of protein and dextrin. Agitation of the solution should therefore be avoided before centrifuging; hence it is best to allow the precipitate to settle in the centrifuge tubes.

The temperature which the solution can tolerate depends on its pH and the amounts of salts present. Hence, if barium hydroxide has been dissolved to a pH of 9.5 the solution must not be kept over night at a temperature higher than 30°, but if the pH is not higher than 8 the temperature may safely be raised to 50° (se below). The solution tolerates 0.1-N barium acetate rather well, but even at 0.2-N some enzyme is destroyed. However, in order to aid precipitation, this high temperature need only to be maintained for a short period, e.g. 5 minutes. It is advisable to heat the solution several times to the high temperature keeping the solution there for a short period and then cooling in water. In this way destruction of enzyme is effectively avoided.

The amount of precipitate and the resistance of the enzymes to heat increase if a solution treated with barium hydroxide in the way described above is saturated with hydrogen sulfide. An enzyme solution which, after treatment with hydrogen sulfide, has been kept at 50° for some short periods can afterwards be kept at this temperature for several days if the pH does not exceed about 7.8. The solution should be kept in a vessel which is only half full and in which the air has been replaced with hydrogen sulfide. Repeated treatment of the cold solution with hydrogen sulfide and subsequent heating to 50° will gradually result in a state where only sulfur is precipitated.

The barium ions remaining in the solution can be precipitated with sulfuric acid in the cold with vigorous stirring; generally a 4-N solution was used. The acid should be added from a capillary tube. The pH should not be allowed to decrease below 7 and the mixture should assume room temperature before centrifuging. The pH of a solution treated in this way normally increases by about one tenth during the next few hours. Hence it is advisable to allow the solution to stand for some hours or over night in order to avoid centrifuging twice. The treatment with sulfuric acid will succeed even if the solution has not been treated with hydrogen sulfide and this method of purification may be of use for industrial purposes. If after its elimination with sulfuric acid more barium hydroxide is dissolved until the solution colours phenolphthalein a rather marked precipitation of protein will occur.

The solution which contains Ba^{++} and is saturated with hydrogen sulfide is then purified by adding alcohol to 30 % by volume. After the addition of alcohol the solution is saturated with hydrogen sulfide, a process which ought to be repeated after a few hours or preferably after a few days. The solution is kept at room temperature. It will not tolerate a temperature higher than 22° for more than a short period. It is advisable to allow the solution to stand saturated with hydrogen sulfide for several days at room temperature. A heavy precipitate of inert material usually appears. Alcohol is then added to the supernatant to 50 % by volume and the solution is again saturated with hydrogen sulfide. The enzymic activity will now be destroyed if the solution is kept at room temperature. However, a suitable temperature for storage while the precipitate settles is 0–6°, but the solution should be allowed to reach room temperature before it is centrifuged. A high pH-value, e.g. over 8, causes precipitation of part of the enzyme. Therefore, if in 50 % alcohol a precipitation of dextrins takes place, the alcohol concentration should be lowered to about 45 %. Whether or not enzymes are lost at this stage depends partly on the pH of the solution (in the presence of Ba^{++}) and partly on the dextrin concentration. If the alcohol concentration is increased over 50 %, most of the amylases are precipitated around 60 %. This is probably due to the presence of the Ba ions, which causes proteins of even low molecular weight to precipitate.

The Ba ions can be easily removed by precipitation with ammonium sulfate: The solution is brought temporarily to room temperature and the sulfate is added in solid form, extremely well pulverized. The solution must be stirred continuously and the quantity of sulfate necessary for precipitation must be tried out carefully; any perceptible excess should be avoided. The precipitate can be centrifuged off after about 30 minutes when it begins to settle (to "loosen" from the surface), but the supernatant should be kept at least over night in the refrigerator for flocculation. The enzymes are now fairly insensitive to alcohol at low temperatures and the alcohol content can be raised to 60 % with only an insignificant loss of activity, provided that the dextrin content is not high enough to cause precipitation in which case enzyme will be precipitated also. In most experiments, however, the precipitation of the enzymes took place from a solution containing 50 % alcohol. This solution is mixed, at a temperature of about 10°, with an equal volume of acetone of room temperature. The mixture should be shaken and now and then raised to room temperature to dissolve the dextrins before centrifuging which should take place within an hour. The precipitate which is syrupy is washed by shaking with 63 % aceton containing sodium acetate and acetic acid (pH = 4.4) at a concentration of about 0.1-N. If the precipitate is a thin and watery syrup after centrifuging the solution should be diluted with 50 % alcohol before adding the acetone in order to reduce the carbohydrate concentration and make the precipitate a rather thick syrup after centrifuging. The volume of acetone should be equal to the volume of the diluted enzyme solution. If the centrifuged precipitate is still syrupy after washing it is shaken again with a fresh quantity of 63 % acetone until it is solid.

The precipitate is then shaken with 55 % acetone containing 0.1-N acetate, pH = 4.4, two or three times until no more is dissolved. After each centrifuging the tube should be placed upside down to drain off all liquid. However, it must be observed that the precipitate absorbs moisture. The 55 % acetone solution should be ice-cold when added but should reach room temperature during the shaking. No loss of enzyme occurs at this stage, even if the preparation is left in contact with the acetone for several days at room temperature. After most of the carbohydrates have been removed in the manner described, remaining salts can be removed by washing with ice-cold, 80 % acid acetone, i.e. acetone + acetic acid of pH = about 5. If the wash liquid is not acid considerable loss of enzyme takes place. In most cases the precipitate was allowed to stand in contact with the acid acetone over night in the cold room. After centrifuging the tube is placed upside down for draining; the precipitate is sucked dry in a vacuum. The enzymes are then brought into solution by treating the precipitate with ice-cold water for an hour. It appears to be impossible to obtain the enzymes free from carbohydrate by precipitation with alcohol or acetone; neither could this be achieved by precipitation with tannic acid or mercury chloride.

The losses of activity and the degree of purity of the enzyme preparations are shown in the table. In the activity assay amounts of the different preparations were used corresponding to 1/16 ml of the raw concentrated extract. 50 ml reaction mixture containing 500 mg starch.

If the precipitation of the enzyme was performed on a solution containing 30 % alcohol the nitrogen content was 0.072 g and the dry-weight about four times as much. This shows the importance of precipitating the enzymes with acetone from a solution containing as much alcohol as possible. The purified enzyme, $Sf_{30^\circ} = 141$, contained about 50 % carbohydrate, determined as glucose after hydrolysis. The high nitrogen content, 29 %, in the final product is probably due to an ion exchange between Ba^{++} and NH_4^+.

The maximum yield of sugar as determined after 23 hours (see table) shows as usual a slight decrease as the degree of purity of the enzyme increases. There are of course many factors which influence the yield of sugar, as for instance the relative amounts of α- and β-amylase in the preparations. In most cases the yield decreases with the age of the starch solution (retrogradation). The composition of the buffer solution appears to have a certain influence. The concentration of the enzyme also plays a role. VOLZ and CALDWELL[3] were able, by using large quantities of Taka amylase, to increase the apparent yield of sugar from 60 to nearly 100 %. With purified malt amylase ($Sf_{30^\circ} = 75$) an increase of the amount of enzyme from 0.21 to 5 mg gave an increase of 8 % up to 83 % reducing sugar calculated as maltose. In the following experiment, however, the concentration of the active enzyme had

Table

	Reaction Time: min.	mg Maltose per 5 ml	Colour with iodine	mg nitrogen in reaction mixture	$Sf_{30°}$[1]
Concentrated raw extract	2	11.1	violet-blue	1.03	1.2
	4	21.1	red-violet		
	8	31.3	yellow	dry-weight = 0.0262 g	
	23 hours	43.7	colourless		
Before purific. with alcohol	2	11.1	violet-blue	0.63	
	4	21.4	red-violet		
	8	33.1	yellow		
	23 hours	43.8	colourless		
After treat. with 50 % alcohol and precip. of Ba with $(H_4N)_2SO_4$	2	9.5	violet-blue	—	
	4	17.9	red-violet		
	8	30.5	red		
	11	—	yellow		
	23 hours	42.5	colourless		
After precip. with acetone and treat. with 63, 55 and 80 % acid acetone and dissolving in water	2	6.4	violet-blue	0.034	141
	4	11.8	violet-blue	dry-weight = 0.000116 g	
	8	21.8	red-violet		
	17	33.6	yellow		
	21 hours	41.1	colourless		

[1] "Saccharifying faculty" as defined by EULER and SVANBERG[2].

no influence on the final sugar formation: An alkaline enzyme solution (pH = 7.9) was diluted in the ratio 1 : 16 with water and kept — after the activity had been determined — for 24 hours at room temperature. After that about 60 % of the activity had been lost but the final yield of sugar was unchanged. Certain unfinished experiments tend to show that the final yield of sugar is increased if a substance present in the malt extract is removed.

The author wishes to express his gratitude for grants from Statens Naturvetenskapliga Forskningsråd and the Hierta-Retzius Scholarship Fund which made this investigation possible.

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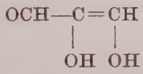
ARKIV FÖR KEMI Band 2 nr 6

Mitgeteilt am 7. Dezember 1949 durch HANS VON EULER

Beiträge zum Redukton-Problem

Von BERND EISTERT

Das durch Erwärmen von Glucose mit verdünnter Alkalilauge erhältliche *Redukton* wurde von EULER und MARTIUS[1] als *Enol-tartronaldehyd*

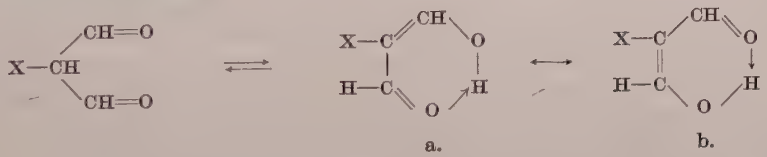


$$\mathrm{OCH{-}\underset{\displaystyle OH}{\underset{|}{C}}{=}\underset{\displaystyle OH}{\underset{|}{CH}}}$$

erkannt. Es ist demgemäss das Oxy-Derivat des Malondialdehyds

$$\mathrm{O{=}HC{-}CH_2{-}CH{=}O} \text{ bzw. seiner Enolform } \mathrm{O{=}CH{-}(COH){=}CHOH}.$$

Bekanntlich sprechen vielen Gründe dafür, dass bei der Enolisierung *offen*kettiger β-Dicarbonylverbindungen, zu denen auch der Malondialdehyd und seine Derivate I gehören, die *cis*-Enolformen entstehen. Diese können nämlich, im Gegensatz zu den trans-Enolformen, eine zusätzliche Stabilisierung dadurch erfahren, dass sie einen ,,Proton-Chelatring'' bilden, den man für die Malonaldehyd-Enole I gemäss II a ⟷ b formulieren kann:



I. II.

Diese Formulierung soll u. a. besagen, dass in solchen ringförmigen Gebilden der Unterschied zwischen Doppel- und Einfachbindungen in ähnlicher Weise ,,ausgeglichen'' erscheint wie etwa im *Benzol*[2], und dass das in ,,Scherenbindung'' zwischen den beiden Sauerstoffatomen befindliche Proton weniger beweglich ist als in nicht-chelatisierten Enolen.

F. ARNDT[3] hat bereits vorgeschlagen, auch das *Redukton* als Chelat aufzufassen; dies entspricht also der Formulierung II (X = OH). Im folgenden soll nun das Verhalten des Reduktons mit dem von anderen, als cis-Enol-Chelate aufgefassten

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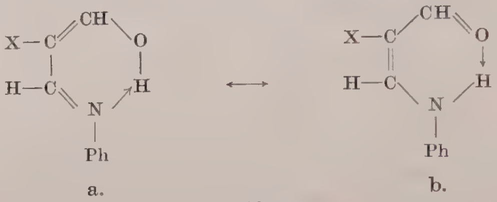
[1] H. VON EULER u. C. MARTIUS, Svensk Kem. Tidskr. *44* (März 1933); Liebigs Ann. Chem. *505*, 73 (1933).

[2] Vergl. B. EISTERT: ,,Tautomerie und Mesomerie" (Stuttgart 1938) S. 169 ff. — Die Auffassung der β-Diketon-Enole als Chelate geht auf SIDGWICK zurück. — Zur Mesomerie des Benzols etc. s. B. EISTERT: ,,Chemismus und Konstitution" Bd. I (Stuttgart 1949) S. 108 ff.

[3] Zitiert von H. v. EULER u. H. HASSELQUIST, Arkiv Kem., Mineral. o. Geol. *26 a*, No. 25, S. 7 (1949).

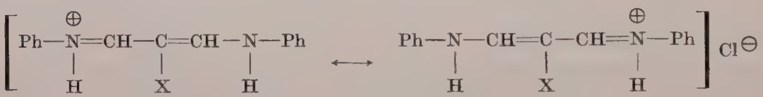
Verbindungen verglichen und die Anwendbarkeit der Chelat-Vorstellung auf das Redukton geprüft werden.

1) Soweit aus der Literatur[4] feststellbar, liefern alle bisher bekannten Malonaldehyde I mit Anilin glatt *Mono*-Anile. Für diese kann man gleichfalls eine Chelat-Formulierung III a ⟷ b anwenden, die sie als „vinylenhomologe Carbonamide" mit in Scherenbindung befindlichem Proton[2] erscheinen lässt:



III.

Diese Mono-Anile gehen nun in den meisten bisher bekannten Fällen beim Behandeln mit überschüssigem Anilin-Hydrochlorid in intensiv gelbe Hydrochloride von *Di*-Anilinoderivaten über, die man vom Standpunkte der Mesomerielehre aus als „vinylenhomologe Amidoniumsalze" aufzufassen hat:



$$\left[\mathrm{Ph{-}\underset{H}{\overset{\oplus}{N}}{=}CH{-}\underset{X}{C}{=}CH{-}\underset{H}{N}{-}Ph} \longleftrightarrow \mathrm{Ph{-}\underset{H}{N}{-}CH{=}\underset{X}{C}{-}CH{=}\underset{H}{\overset{\oplus}{N}}{-}Ph}\right]\mathrm{Cl}^{\ominus}$$

a. b.

IV.

Bei dieser Umwandlung in die gelben Salze IV wird also der Chelatring von III geöffnet und die enolische OH-Gruppe durch eine Phenylammoniumgruppe substituiert. Die Salze IV spalten indessen bereits mit heissem Wasser mehr oder weniger leicht wieder Anilin-Hydrochlorid ab, indem sie die Mono-Anile III zurückbilden. Die Natur des Substituenten X scheint dafür massgebend zu sein, ob die Konjugationsenergie, die bei der Bildung von IV gewonnen wird, grösser oder kleiner ist als die Bildungsenergie des Chelatringes III und des Anilin-Hydrochlorids.

Wir überzeugten uns am Beispiel des relativ leicht zugänglichen[4] *Brom*-Malonaldehyd-Monoanils III ($X = Br$) vom glatten Verlauf und der Reversibilität der Umsetzung $III + PhNH_3Cl \rightleftarrows IV$.

Das von uns nach den etwas spärlichen Angaben von DIECKMANN und PLATZ[5] aus der sogen. Mucobromsäure[6] über das Salz IV ($X = Br$) hergestellte Brommalonaldehyd-Monoanil zeigte den Schmp. 164° (ohne Zers.), während die genannten Autoren 184° (Zers.) angeben. Unser Präparat zeigte jedoch im übrigen die angegebenen Eigenschaften und gab auch stimmende Analysenwerte, sodass wir vermuten, dass hier in der Originalarbeit ein Druckfehler unterlaufen ist.

Aus dem *Redukton* II ($X = OH$) dagegen liess sich bisher, entgegen anderslautenden Angaben englischer Forscher[7], auch mit überschüssigem Anilin-Hydrochlorid nur ein *Mono*-Anil III (X = OH) bzw. dessen Hydrochlorid gewinnen. Verschiedene Variationen der Versuchsbedingungen, insbesondere der zugesetzten Säuremenge, änderten daran nichts[8]. Die OH-Gruppe in Stellung 2 scheint also die Bildung des „vinylenhomologen Amidoniumsalzes" IV zu verhindern bzw. den Chelatring des Monoanils III zu stabilisieren. (Siehe „Zusatz" auf S. 5.)

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[4] Siehe z. B. W. DIECKMANN u. L. PLATZ, Ber. deutsch. Chem. Ges. *37*, 4640 (1904); G. SCHWARZENBACH u. Mitarb., Helv. chim. Acta.

[5] a. a. O. p. 4645/46.

[6] „Organic Syntheses" vol. *27*, p. 60.

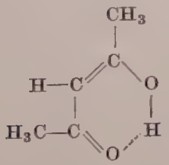
[7] H. S. FORREST u. J. WALKER, Nature *161*, 308 (1948); J. Chem. Soc. *1949*, 79 u. 2002.

2) Das *Redukton* gibt bekanntlich[1] mit alkohol. $FeCl_3$-Lösung eine nur sehr kurze Zeit beobachtbare, intensiv blau*grüne* Farbreaktion, die für alle „Endiole" charakteristisch zu sein scheint[9]. Sie beruht vermutlich auf der Bildung eines merichinoiden Chinhydron-Eisenkomplexes[10], der alsbald unter Reduktion des Fe(III)-Ions in Oxydationsprodukte des Endiols zerfällt.

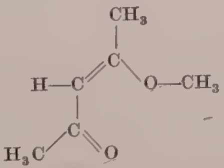
Wir fanden nun, dass eine mit *Ammoniak* versetzte wässr. Lösung von Redukton bei Zugabe von $FeCl_3$ eine intensiv rot*violette* Lösung gibt, die längere Zeit beständig ist und erst allmählich unter Braunfärbung zersetzt wird.

Eine ganz entsprechende Farbreaktion gibt, wie wir weiter feststellten, auch das Redukton-*Monoanil* III (X = OH); hier ist die Färbung etwas blauer violett, aber ähnlich beständig. Man wird daher annehmen dürfen, dass sich aus Redukton und Ammoniak, wenigstens i. Ggw. von $FeCl_3$, ein dem Anil III analoges „Mono-Imin" (III, X = OH, H anstelle von Ph) bildet. Über die Konstitution der violetten Reduktonimin-Eisenkomplexe sind noch keine Angaben möglich. Jedenfalls ist dafür die Anwesenheit der OH-Gruppe in Stellung 2 notwendig, denn das *Brom*malonaldehyd-Monoanil III (X = Br) gibt mit $FeCl_3$ keine Farbreaktion. — Freier Brommalondialdehyd wird von $FeCl_3$ intensiv violettrot gefärbt[5].

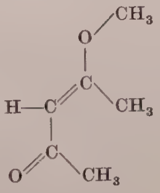
3) Cis-Enol-Chelate werden von abs.-ätherischer *Diazomethan*-Lösung in Abwesenheit OH-gruppenhaltiger „Aktivatoren" nicht oder nur sehr langsam in den entspr. Enolmethyläther umgewandelt.[11] Wir überzeugten uns durch einen Versuch mit *Acetylaceton*, dass dessen cis-Enol-Chelat V, wie es in Ätherlösung anzunehmen ist, in Abwesenheit von Methanol ebenfalls von Diazomethan nicht oder höchstens sehr langsam methyliert wird:



V.



V a.



V b.

Eine Lösung von Acetylaceton in abs.-ätherischer Diazomethan-Lösung bleibt lange Zeit unverändert. Bei Zusatz von Methanol dagegen erfolgt unter Stickstoff-Entwicklung die Bildung des (nicht-chelatisierten) Enol-Methyläthers bzw. eines Gemisches der beiden stereomeren Methyläther V a und V b. Über den Einfluss des Methanols auf die sterische Struktur Enoläther der wird an anderer Stelle publiziert werden.

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[8] H. v. Euler u. H. Hasselquist (im Druck).

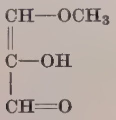
[9] Sie wird z. B. auch vom Cumarindiol und vom 1-Thiacumarindiol gegeben (Privatmitteilung von Hrn. F. Arndt — Istanbul).

[10] Vergl. hierzu L. Michaelis u. S. Gramick, J. Amer. chem. Soc. *70*, 624 (1948); R. Criegee u. K. Klonk, Liebigs Ann. Chem. *564*, 4 (1949).

[11] A. Schönberg u. A. Mustafa beschrieben diese Erscheinung bei chelatisierten aromatischen o-Oxycarbonylverbindungen vom Typus des Salicylaldehyds usw. (J. chem. Soc. London *1946*, 746). An rein aliphatischen Enolen hat man sie m. W. noch nicht beschrieben.

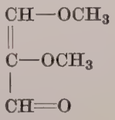
Dass das *Redukton* (II, X = OH) durch Diazomethan bereits *ohne* Methanolzusatz ziemlich rasch zum Monomethyläther VI methyliert wird[1], beruht offenbar darauf, dass es selbst die ,,aktivierende" OH-Gruppe (in Stellung 2) enthält. — Über Erfahrungen bei der Einwirkung von Diazomethan auf Mono-Anile III siehe unten.

Bei der Einwirkung *überschüssigen* Diazomethans in Äther-Methanol-Lösung erhält man aus *Redukton* ein öliges Produkt, das bisher nicht näher untersucht wurde.[12] Es ist sehr wahrscheinlich, dass dieses Öl aus dem Dimethyläther VII und n. U. Stereomeren besteht, denn es ist bekannt[13], dass (cyclische) ,,Endiole" von Diazomethan zunächst an der ,,enolischen" und dann auch an der benachbarten ,,alkoholischen" OH-Gruppe methyliert werden, die ja ihrerseits durch die benachbarten CO-Gruppen ,,acidifiziert" ist[14]. Es ist jedoch durchaus möglich, dass daneben auch die *Aldehyd*gruppe mit Diazomethan reagiert, wobei die Bildung von ,,Äthylenoxyd" VIII bzw. ,,Methylketon" IX zu erwarten wäre[15]. Hierüber wird erst eine nähere Untersuchung jenes Öles Klarheit bringen.



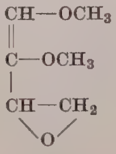
$$\begin{array}{l} CH{-}OCH_3 \\ \| \\ C{-}OH \\ | \\ CH{=}O \end{array}$$

VI.

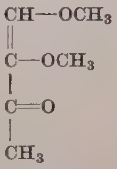


$$\begin{array}{l} CH{-}OCH_3 \\ \| \\ C{-}OCH_3 \\ | \\ CH{=}O \end{array}$$

VII.



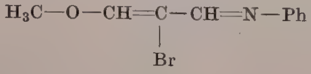
VIII.



$$\begin{array}{l} CH{-}OCH_3 \\ \| \\ C{-}OCH_3 \\ | \\ C{=}O \\ | \\ CH_3 \end{array}$$

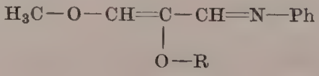
IX.

Lässt man Diazomethan in abs.-ätherischer Lösung auf das *Brom*malonaldehyd-Monoanil III (X = Br) einwirken, so beobachtet man keine N_2-Entwicklung. Der Chelatring verhindert also auch hier die Methylierung der enolischen OH-Gruppe. Fügt man jedoch Methanol hinzu, so erfolgt allmähliche Umsetzung, wobei die Kristalle des Anils unter N_2-Entwicklung in Lösung gehen. Beim Einengen der Lösung im Vacuum bei gew. Temperatur scheiden sich Blättchen vom Schmp. 154° aus, die aus dem Methyläther X bestehen. Die Hauptmenge des Reaktionsproduktes ist ein Öl, das sich am Licht rot verfärbt.



$$\begin{array}{c} H_3C{-}O{-}CH{=}\underset{\displaystyle Br}{\underset{|}{C}}{-}CH{=}N{-}Ph \end{array}$$

X.



$$\begin{array}{c} H_3C{-}O{-}CH{=}\underset{\displaystyle O{-}R}{\underset{|}{C}}{-}CH{=}N{-}Ph \end{array}$$

XI.

Das *Redukton*-Monoanil III (X = OH) reagiert erwartungsgemäss bereits ohne Methanolzusatz allmählich mit äther. Diazomethanlösung; nach Zugabe von Methanol ist die Umsetzung in kürzerer Zeit beendet. Beim Einengen der Lösung im Vacuum hinterbleibt ein Gemisch von Kristallen und Öl. Die Kristalle zeigten nach dem Umkristallisieren den Schmp. 118—119° (Zers.). Der Mischschmelzpunkt mit dem Ausgangsstoff lag bei 75—90° (Zers.). Es handelt sich vermutlich um den Monomethyläther XI (R = H). Die öligen Anteile dürften auch den Dimethyläther (XI, $R = CH_3$) und stereomere Äther enthalten.

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[12] H. v. Euler u. H. Hasselquist, Reduktone. Saml. chem. u. chem.-techn. Vorträge, 50, 1950.

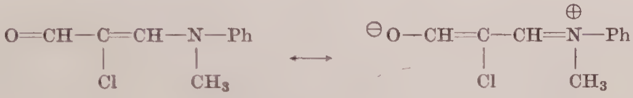
[13] F. Arndt u. B. Eistert, Ber. deutsch. Chem. Ges. *62*, 36 (1929); *68*, 1572 (1935).

[14] ,,Tautomerie und Mesomerie"[4] S. 39; ,,Chemismus und Konstitution" Bd. I, S. 199 ff.

[15] F. Arndt u. B. Eistert, Ber. deutsch. Chem. Ges. *68*, 196 (1935).

Analyse:	$C_{10}H_{11}O_2N$	Gef. CH_3O—	16,6 %
	(Mol. Gew. 177)	Ber.	17,5 %

Bei der Umsetzung von *Chlor*malonaldehyd mit *Monomethylanilin* erhielten DIECKMANN und PLATZ[16] eine farblose Verbindung, die sie als 1-Phenylmethylamino-2-chlor-acrolein XII a formulierten. Diese Verbindung ist vom Standpunkt der Mesomerielehre aus als „homologes Carbonamid" gemäss XII a ←→ b aufzufassen:



$$\underset{\text{a.}}{O{=}CH{-}\underset{|}{\overset{}{C}}{=}CH{-}\underset{\underset{CH_3}{|}}{N}{-}Ph}\ \longleftrightarrow\ \underset{\text{b.}}{{}^{\ominus}O{-}CH{=}\underset{|}{C}{-}CH{=}\overset{\oplus}{\underset{\underset{CH_3}{|}}{N}}{-}Ph}$$

Cl (an C in a. und b.)

a. b.

XII.

Die Verbindung ist, als nicht-chelatisierter Dipol, in Alkohol sehr viel leichter löslich als das entsprechende methylfreie Chelat III ($X = Cl$). Es wird von Interesse sein, die Umsetzung von *Redukton* mit Monomethylanilin und anderen sekundären Basen zu studieren. Vorversuche ergaben, dass eine Umsetzung eintritt.

Die ursprüngliche Absicht, im Brom-Malonaldehyd-Monoanil das Brom-Atom gegen die OH-, OCH_3- oder eine andere Gruppe auszutauschen und so zu Derivaten des Reduktons zu gelangen, liess sich bisher noch nicht verwirklichen; das Brom ist hier recht fest gebunden. Die Versuche sollen jedoch fortgesetzt werden. Versuche, das Natrium-Salz des Reduktons mit Dimethylsulfat zu methylieren, haben nur ein nicht kristallisierendes Öl ergeben.

Die bisher vorliegenden experimentellen Tatsachen lassen sich jedenfalls mit den Chelat-Formeln II bzw. III ($X = OH$) für das Redukton bzw. sein Mono-Anil recht gut verstehen, wenn auch das bisherige Scheitern der Versuche zur Herstellung der „vinylenhomologen Amidoniumsalze" IV noch einer ausreichenden Erklärung bedarf. Auch wenn man anstelle des Chelatringes eine offene trans-Figuration annähme, würde damit die Nicht-Umsetzbarkeit mit weiterem Anilin-Hydrochlorid keineswegs verständlicher sein. Ein Vergleich des Reduktons mit solchen analogen Endiolen, die durch Einbau der Gruppe —C(OH) = C(OH)—CO— in einen Ring in der nicht-chelatisierbaren trans-Figuration festgelegt sind (z. B. Cumarindiol[13], Dihydropyrogallol[17] u. a.), wäre auch in dieser Hinsicht interessant.

Ich danke Herrn Prof. Dr. HANS VON EULER und Svenska Institutet aufrichtig für die grosszügige Einladung, die es mir ermöglichte, näheren Einblick in die Probleme der Endiol-Chemie zu nehmen. Hrn. Ing. HANS HASSELQUIST und Hrn. ARMIN GLASER danke ich sehr für ihre Unterstützung bei den beschriebenen Versuchen.

Zusatz bei der Korrektur (13.III. 1950):

Inzwischen wurde ermittelt, dass auch der 2-*Acetamido*-malondialdehyd ($X = CH_3CO{-}NH{-}$) nur ein *Mono*-Anil vom Typus III bildet[18]), während sowohl vom *un*substituierten Malondialdehyd ($X = H$)[19] wie auch vom Malondialdehyd-

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[16] a. a. O. p. 4645.

[17] B. PECHERER, L. M. JAMPOLSKY u. H. M. WUEST, J. Amer. chem. Soc. *70*, 2587 (1948).

[18] J. W. CORNFORTH, E. FAWAZ, L. J. GOLDSWORTHY u. R. ROBINSON, J. chem. Soc. *1949*, 1549.

[19] R. HÜTTEL, Ber. deutsch. Chem. Ges. *74*, 1825 [1941].

2-carbonsäureester (X = H_5C_2OOC—)[20] nur *Di*-Anile bzw. deren Salze vom Typus IV bekannt sind, und die *2-Halogen*-malondialdehyde sowohl Mono-Anile III als auch Dianile bzw. deren Salze IV bilden. Es ergibt sich also folgende Beziehung:

X	E-Effekt[21]	Mit Anilinhydrochlorid bilden sich:
H_5C_2OOC—	—E	Dianile bzw. deren Salze IV
H—	Null	» » » » »
Halogen	schwach + E	In reversibler Reaktion Monoanile III und Dianile IV
HO—	+E	Nur Monoanile III
CH_3—CO—NH—	+E	» » »

Man wird aus dieser Zusammenstellung folgern dürfen, dass Substituenten X, die einsame Elektronenpaare besitzen und einen + E-Effekt ausüben können[21], das Zustandekommen des Dianils bzw. seines Salzes IV hindern; die Halogene mit ihrem nur geringen + E-Effekt bilden das Zwischenglied zwischen den extremen Gruppen. Das Verhalten des Triose-Reduktons verliert damit seine Ausnahmestellung.

Stockholm, Universität, Inst. f. organ.-chem. Forschung und Vitamininstitut.

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[20] L. PANIZZI, Gazz. chim. Ital. *76*, 56 [1946].
[21] „Chemismus und Konstitution"[4] S. 131.

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Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 7

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Investigations on malt amylase

V. On the stability of α-amylase by lack of calcium

By Eskil Hultin

With 5 figures in the text

It has been known for a long time that calcium salts have a stabilizing effect on malt α-amylase (1-4). Therefore in most investigations the amount of calcium salts necessary to give maximum stability is added to the amylase solution. However, only very little attention has been paid to the behaviour of unstabilized amylase. This investigation was carried out to provide information on the subject.

The stabilizing effect of calcium salts

Some amylases require the presence of calcium ions for the maintenance of their activity, and on addition of oxalate or fluoride ions their activity is reduced (5). When α-amylase is extracted from malt, the crude solution contains calcium salts in quantities sufficient to give fairly good stability at ordinary temperatures, but not always sufficient to give such stability that the extract can be heated to 70° at pH 6.5 without appreciable losses. However, if calcium salts have been almost completely eliminated from an amylase solution by precipitation with potassium oxalate, the activity of the enzyme will decrease continuously and rapidly even at room temperature. If a sufficient amount of calcium ions is added, this rapid decrease in activity will stop, but the amylase already inactivated will not recover its activity. This is demonstrated by the following experiments.

A dry preparation of malt α-amylase was made in the following way.[1] A malt extract was heated to 70° C for 10 minutes at pH 6.5, after addition of calcium chloride, and filtered. The solution was cooled to 0° C and acidified to pH 4.5, whereupon a 10 % tannic acid solution was added until no increase of the precipitate was noticed upon further addition of the reagent. The precipitate was centrifuged off and extracted with acetone, sucked off, washed with ether and dried in vacuo.

25 ml of a centrifuged 2 % extract of this amylase preparation were mixed with 0.5 ml 1 N calcium chloride solution, 2 ml saturated potassium oxalate solution (3 N), 5 ml 4 N acetate buffer solution of pH 5.3, and made up to 50 ml with water.

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[1] After these experiments were finished, an investigation on the purification of malt α-amylase with tannic acid was carried out, the results of which — including a better procedure — will soon be published in this journal.

The activity of the solution was measured viscosimetrically (6, 7) whereby distilled water was used for the dilution of the samples to a suitable strength. A sample taken immediately had an activity of 260 μA/ml. After 110 min the activity was 79 μA/ml, and after 170 min 31 μA/ml.

From a similar mixture two samples of 10 ml were withdrawn immediately after the mixing and 2 ml 1 N calcium chloride solution were added. One sample was measured immediately and the other after 2 hours, and the measurements of amylase activity were 229 and 230 μA/ml respectively. A third sample of 10 ml was taken 2 $^1/_2$ hours after the mixture was made up. 2 ml 1 N calcium chloride solution were added. The activity was 34 μA/ml.

All these experiments were carried out at room temperature except the activity determinations, which were made at 30.0° C.

From these experiments we conclude that samples withdrawn from unstable amylase solutions for activity assays can be stabilized immediately by addition of calcium salts and that the inactivation of the amylase otherwise proceeds so rapidly that the results of the assay will be rather inaccurate.

However, it is possible to make assays of unstable enzyme solutions if, in the calculations, the expression $(1 - e^{-kt})/k$, where k is the rate constant for the inactivation of the enzyme and t is the time, is substituted for the time (8). But it is of course preferable to stabilize the enzyme in all cases when it is possible.

The influence of acidity

The stability of malt α-amylase is considerably influenced by the acidity of the medium. Ohlsson (9) studied the spontaneous inactivation of the amylases in crude malt extracts, which — as already stated — are normally fairly well stabilized by calcium salts dissolved out of the malt meal.

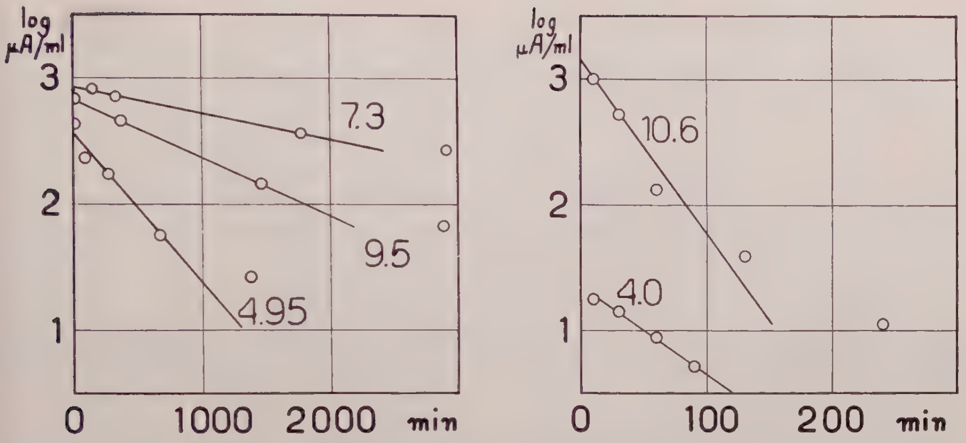
This investigation gives the influence of the pH on the stability of the α-amylase in the presence of an excess of oxalate. This reagent lowers the calcium ion activity tremendously.

The reaction mixtures were made up of 25 ml of a centrifuged 2 % extract of the amylase dry preparation described, 1 ml 0.5 N calcium chloride solution, 2 ml buffer solution (described below), 20 ml distilled water and 2 ml saturated potassium oxalate. In some experiments a weaker amylase solution was used.

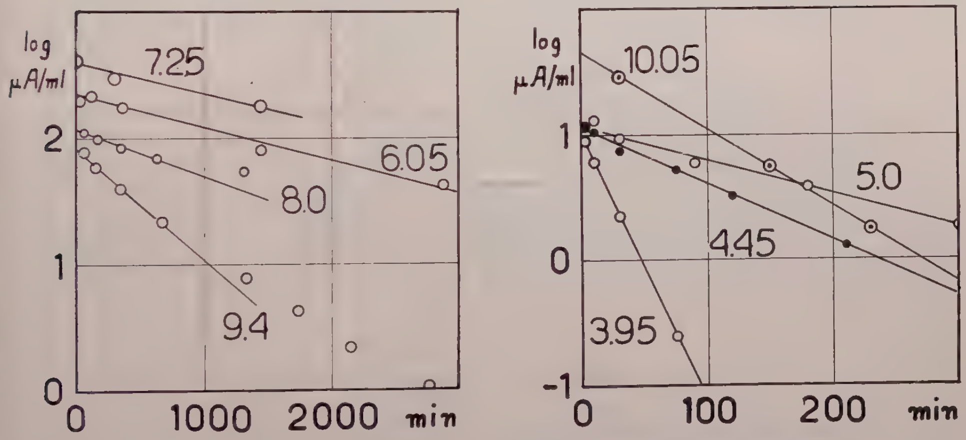
Buffer solutions were made up of suitable amounts of 4 N sodium acetate and 4 N acetic acid solutions for pH values lower than 5.5, and of 0.3 M potassium dihydrogen phosphate and 0.3 M disodium phosphate for pH values between 5.5 and 8. For higher pH values ammonia was added to the solution.

The inactivation experiments were carried out at 0° and at 30° C. The flasks, in which the mixtures were made up, were immersed in the thermostat when all ingredients except the potassium oxalate had been added. After the temperature had equalized, the potassium oxalate solution, which had the same temperature, was added and the flasks shaken. 5 ml samples were withdrawn at suitable intervals and for stabilization pipetted into test tubes which contained 2 ml 0.5 N calcium chloride solution, whereupon the activity was assayed.

The results of the experiments at 0° are given in Figs. 1 and 2 and those at 30° in Figs. 3 and 4. In these graphs the logarithm of the remaining activity is plotted against time. For a reaction of the first order the points then lie on a straight line. The shape of the curves indicate that under the given experimental conditions the



Figs. 1 and 2. Inactivation of α-amylase at 0° C and various pH.



Figs. 3 and 4. Inactivation of α-amylase at 30° C and various pH.

inactivation of the amylase proceeds approximately as a reaction of the first order during the deterioration of the main part of the amylase, but the last traces of the amylase seem to be comparatively resistant.

The shape of the curves also implies that the reactions, by which calcium is eliminated from and built into the active part of the amylase molecule, take place instantaneously.

If we calculate the half life period of the amylase using the initial parts of the curves, we get figures valid for the inactivation of the main part of the enzyme. Although these figures are not very accurate, they make it possible for us to compare the stability of the calcium-free amylase with that of normal amylase solutions. In Fig. 5 the half life period of the calcium-free amylase — calculated in this way from Figs. 1–4 — and the corresponding values of stable amylase at 38° calculated by Ohlsson (9) are put together. On comparing the results of these investigations

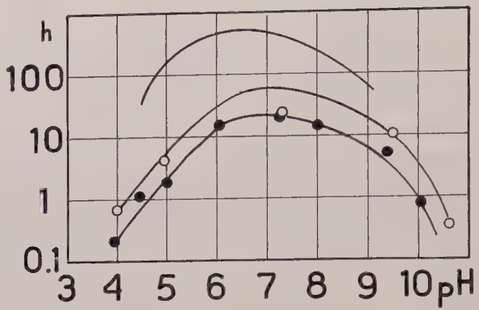


Fig. 5. The half life period of α-amylase in hours at 38° C according to Ohlsson (top curve) and — in the presence of potassium oxalate — at 0° (middle curve) and at 30° (bottom curve).

we find that the stability of malt α-amylase is — roughly speaking — only 1/50 to 1/100 of that of a common crude malt extract. The stability is very much influenced by the pH. At pH = 7 the half life period is about 10 times that at pH = 5 or 9.5. The inactivation of the enzyme proceeds about three times as rapidly at 30° as at 0° C.

The inactivation of α-amylase in β-amylase preparations

The instability of malt α-amylase in the absence of calcium salts, especially at low pH values, simplifies the inactivation of α-amylase in preparations of malt β-amylase. The common procedure includes inactivation of the α-amylase by acidifying the solution to about pH 3.3–3.6 for some time (9, 10). As β-amylase does not require calcium salts for its stability it is best first to precipitate calcium by adding oxalate.

The inactivation of α-amylase on dialysis

Several scientists have noticed that malt amylase looses part of its activity on dialysis. Lisbonne and Vulquin (11) found that a long electrodialysis reduces the activity of malt amylase considerably, although its saccharifying activity does not disappear completely. Ohlsson (9) found that on dialysis of malt extract the α-amylase disappears but not the β-amylase. Hamoir (12) tried to purify malt α-amylase by electrodialysis but the losses of the enzyme at the treatment were very large. Kneen, Sandstedt, and Hollenbeck (4) showed that the stability of malt α-amylase was lowered on dialysis against distilled water and increased on dialysis against tap water.

The losses of amylase on dialysis against distilled water can be explained in two ways. One reason is that the calcium salts are easily dialysed away, whereby the proportion of calcium-free amylase increases, and the amylase shows a lower stability. Another reason for the losses is that the amylase can pass through collodion membranes. In earlier times collodion bags were usually employed for dialysis, and a good deal of the earlier difficulties in dialyzing this enzyme must be due to unsuitable membranes. Long ago Fränkel and Hamburg (13) made some experiments on the ability of malt amylase to go through dialysis bags. They have stated

— unlike later observations — that the saccharifying diastases pass through the membranes, whereas the liquefying ones remain within the bags. The ability of malt α-amylase to go through collodion membranes has recently been employed in the purification of malt α-amylase by means of ultrafiltration, as reported by DANIELSSON and SANDEGREN (14).

I have made some experiments on the dialysis of the amylase in collodion bags and in cellophane bags and found that it can easily be shown by activity determinations that the enzyme is present in the water, against which the enzyme was dialyzed in collodion bags, whereas it does not pass cellophane membranes in detectable quantities.

The great losses of activity on electrodialysis are probably due to the effective removal of calcium salts and simultaneous lowering of the pH of the solution towards and even past the iso-electric point of the main malt albumin, which accompanies the amylase in large quantities in crude extracts. The iso-electric point of that malt albumin was determined by LÜERS and LANDAUER (15) as pH = 4.6. At this pH the half life period of the calcium-free amylase is only about 10^{-3} to 10^{-4} compared with the half life period at maximum stability.

The stabilizing effect of some salts

A great many investigations on the influence of various salts on amylases have been reported (16, 17). In the previous chapters it has been pointed out that calcium ions have a great influence on the stability of malt α-amylase. It is reasonable to presume that the stabilizing power of calcium ions is effected so that calcium is in some way chemically bound to a part of the molecule, essential for the stability of its active part, and that this compound deteriorates with much less velocity than the calcium-free amylase. The proportions of this compound and the calcium-free amylase in a solution then depend on the calcium ion activity in the solution and the affinity of calcium to the amylase. We must presume that if other metal ions can stabilize the amylase in the same way, the various metal ions have different affinities to the amylase and the various compounds have different stabilities. Therefore, in investigating the influence of a certain salt on the stability of the amylase, we ought to eliminate other ions with an influence on the stability, in order to avoid a competitive effect, which may render the interpretation of the results difficult or impossible. As calcium ions have a considerably stabilizing effect even in low concentrations and always occur in malt extracts, it is necessary to eliminate them first when the effects of other salts are studied.

The following experiments were carried out on the stabilizing effect of some salts on malt amylase.

0.2 g amylase preparation were dissolved in 50 ml water and ammonia added to pH = 7.5. Then 1 ml 0.5 N calcium chloride solution and 1 ml 3 N potassium oxalate solution were added. The mixture was centrifuged after half an hour and kept at 0° C. It was used for stability experiments for two or three days. Samples of 5 ml of this amylase solution were mixed with 2 ml of water or 0.5 N salt solution and 3 ml of 0.2 N acetate buffer with pH 4.3 and immersed in a thermostat of 30° C. For the activity determinations 2 ml of the mixtures were pipetted into 3 ml of 0.4 % calcium acetate solution. The activity assays were carried out colorimetrically (18).

Table 1

The stabilizing effect of some salts.

Salt	Age of amylase in days	pH	Activity, mg starch/min · ml		
			0 min	30 min	60 min
$SrCl_2$	0	4.30	46	46	46
$CaCl_2$	0	4.30	46	44	46
$Pb(NO_3)_2$	1	4.30	32	10	4
$Cd(NO_3)_2$	1	4.30	33	32	30
$BaCl_2$	1	4.30	38	31	24
Li_2SO_4	1	4.43	30	0	0
$ZnCl_2$	0	4.28	64	26	13
$MnSO_4$	0	4.28	68	29	13
$NiCl_2$	1	4.28	30	3	3
$MgCl_2$	1	4.32	41	5	4
$CoCl_2$	2	4.46	34	5	4
$FeSO_4$	2	4.43	22	0	0
(water)	2	4.46	11	0	0
$CdSO_4$	0		75	46	42
$Ba(NO_3)_2$	0		84	73	67
$Pb(NO_3)_2$	0		93	0	0
(water)	0	4.40	93	8	7
$MnSO_4$	0	4.40	125	71	45
$ZnSO_4$	0	4.40	116	13	8

These preliminary experiments indicate that strontium has about the same stabilizing effect as calcium and also that the stabilizing effect of barium is very great. Cadmium, manganese, and zinc stabilize the amylase appreciably, whereas the value of sodium and potassium (which were added as sodium acetate and potassium oxalate), lithium, magnesium, iron, cobalt, nickel, and lead is none or doubtful.

SUMMARY

The inactivation of a malt α-amylase solution, from which the calcium salts have been precipitated as oxalate, proceeds approximately as a reaction of the first order but only during the deterioration of the main part of the amylase. The deterioration velocity of an amylase solution is increased about 50 to 100 times when the calcium salts are eliminated in this way. The reactions by which calcium is eliminated from and built into the amylase molecule are instantaneous. Calcium-free amylase solutions can be stabilized by calcium, strontium and barium salts. Cadmium, manganese and zinc also have a stabilizing effect on the amylase, whereas lithium, sodium, potassium, magnesium, iron, cobalt, nickel and lead salts show no appreciable stabilizing effect. The losses of amylase on dialysis are due to its ability to go through collodion membranes (but not cellophane) and to the removal of calcium salts, possibly accompanied by a lowering of the pH.

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Investigations on malt amylase

VI. On the purification of α-amylase with tannic acid and acetone

By ESKIL HULTIN

With 6 figures in the text

If tannic acid is added to a malt extract, a precipitate is formed, containing a compound of amylase and the reagent. TRUNKEL (1) found that tannic acid could be extracted from a similar compound with glue by means of alcohol. Tannic acid can be extracted from its compound with malt proteins by acetone, whereupon the amylase can be dissolved in water, as shown by WEIDENHAGEN (2, 3). HOLMBERGH (4) found that the amylase is soluble even in the presence of tannic acid if the solution contains a suitable amount of alcohol. Some experiments on the purification of α-amylase in crude malt extracts have recently been published by HOLMBERGH and HULTIN (5).

This investigation deals with the purification of malt α-amylase with tannic acid and acetone, using a malt extract which has been partly purified by heating to about 70° C.

Preparing the malt extract

It has been reported from several investigations that the α-amylase can be dissolved out of malt meal very easily and rapidly, and that it is best to use water containing calcium salts for the extraction (4, 6–8). This is quite in agreement with my experience that half an hour is sufficient for the extraction and that there is a slight increase in impurities on prolonged extraction. QUENSEL (9) found in his investigation on barley globulins that when a sufficient quantity of the salts has been eliminated by dialysis — so that the conductivity of the solution corresponds to about 10^{-3} N sodium chloride solution — the solubility of the various globulins in water is greatly influenced by the salt content. If the salt concentration is of that magnitude, a slight decrease in salt concentration causes a considerable decrease in the solubility of the globulins. Hence it seems suitable to use calcium salt solutions of about that concentration for the extraction and to add more calcium chloride only after the extract has been separated from the meal. However, such large quantities of salts are usually extracted from the malt meal with reasonable quantities of distilled water that the conductivity of the extract is considerably raised, and later experiments have shown that the preparations contain a good deal of globulins, precipitable by dialysis against a 0.0001 N calcium chloride solution.

Inhibition of the proteolytic enzymes

A crude malt extract contains proteolytic enzymes, which cause a breakdown of the various other proteins present. Thereby the solubilities in ammonium sulphate solutions of the α-amylase and of the other proteins are rendered more similar to one another, which makes the purification of the enzyme by fractionated precipitation with this reagent more difficult (10). These proteolytic enzymes can be inhibited by bromate and iodate (11—13) and the following experiments were made to give an idea on the amounts necessary.

The proteolytic activity was assayed by Ayre and Anderson's method (14). 10 g malt meal were mixed with 25 ml water (bromate or iodate solution was substituted for part of the water in most experiments) and 25 ml acetate buffer solution (6 ml acetic and 13.6 g $NaAc \cdot 3H_2O$ dissolved in water to 500 ml). The mixture was immersed into a thermostat at 45° C and stirred every 5 minutes. After 1 hour, 10 ml of a 30 % trichloroacetic acid solution was added, the mixture was stirred and left to cool. It was then centrifuged and filtered. A similar mixture was left for 3 hours before the addition of the trichloroacetic acid. The nitrogen content in 2 ml of the filtered solution was determined by Kjeldahl's method, mainly by the procedure recommended by Cole and Parks (15), but without selenium in the catalyst mixture.

The results of the experiments are given in Table 1.

Table 1

The activity of the proteolytic enzymes in malt.

Admixture	mg N		
	after 1 hour	after 3 hours	difference
30 mg cystein	3.24 3.16	4.11 4.05	0.88
no admixture	2.49 2.44	3.06 3.04	0.58
2 ml 1 ‰ bromate	2.15 1.96	2.41 2.41	0.40
5 ml 1 ‰ bromate	2.03 2.02	2.15 2.10	0.10
2 ml 1 ‰ iodate	1.92 1.82	2.45 2.31	0.51
5 ml 1 ‰ iodate	1.88 1.88	2.11 2.05	0.20

Some experiments were also made to indicate whether the stability of the amylase is influenced by bromate. Two malt extracts made in the same way, using for one a 0.05 N calcium chloride solution and for the other a 0.05 N calcium chloride solution containing 0.01 % potassium bromate, were preserved for nine days under toluene in a thermostat at 30°. They had the same activity after that period as they had immediately after being made.

As a result of these experiments, a 0.002 N calcium chloride solution, containing 0.01 % potassium bromate, was normally used for the extraction of malt meal.

Coagulating proteins by heat

If a malt extract is heated to 70° at a pH of about 6.5, the β-amylase (16) and the proteolytic enzymes (17) are destroyed but not the α-amylase, and a good deal of the proteins present are coagulated (18, 19). If the extract is heated to a higher temperature, still more protein is coagulated (20) but the stability of the amylase is diminished very much if the temperature is raised above 70°. In order to render the losses of the α-amylase as small as possible, it is important to add enough calcium chloride and to adjust the pH to a suitable value. The following experiment was carried out to check the optimum pH for stability (16).

Malt extract No. 5. 100 g malt meal were extracted first with 400 ml of a 0.01 N $CaCl_2$ solution containing 0.01 % bromate, and after centrifuging with another 250 ml of this solution. The pH of the extract was adjusted to 7.1 and the solution heated to 74° C, cooled and filtered. The pH was then 6.6 and the activity — determined colorimetrically (21) — 45 mg starch/min · ml. (1 mg starch/min · ml corresponds to 0.14 mg maltose/min · ml.) Samples of 25 ml were taken and various amounts — not more than 0.2 ml — of 0.5 N HCl or 0.5 N NaOH were added. The samples were then immersed into a thermostat at 74° for 30 min, after which the pH and the activity were measured. The results are given in Fig. 1.

The best calcium salt concentration when heating a malt extract was the subject of the next few series of experiments.

A series of malt extracts No. 6–9 were prepared in a similar manner using for the extraction a 0.01 % $KBrO_3$ solution containing 0.001 N, 0.005 N, 0.02 N and 0.1 N $CaCl_2$ respectively. After the pH had been adjusted to 7.1 the solutions were heated to 70°, whereby the pH changed to 6.9, 6.8, 6.7 and 6.5 respectively. Three samples were taken of each extract and suitable amounts of 0.5 N HCl or 0.5 N NaOH added, whereupon the samples were heated to 70° C for 30 min. After they had cooled down, the pH and the activity were measured. The results are given in Table 2.

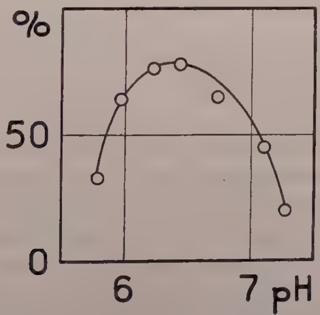

Fig. 1. The percentual remaining α-amylase activity after heating to 74° C.

Table 2

The stability of α-amylase at 70° C in the presence of various amounts of $CaCl_2$. Activity in mg starch/min · ml. The activity before the second heating is given in the first line.

0.001 N		0.005 N		0.02 N		0.1 N	
pH	Activity	pH	Activity	pH	Activity	pH	Activity
	39		45		55		51
6.43	16	6.48	20	6.20	34	6.17	40
6.58	17	6.62	22	6.43	40	6.52	45
6.74	9	6.80	20	6.80	26	6.90	43

These experiments indicate that it is advisable to make the solution 0.02 to 0.1 N as regards calcium salts and to adjust the pH preliminarily to about 6.6 to 6.9 before heating as it will usually decrease somewhat. As the stability is rather dependent on the pH, it is best to adjust the pH to about 6.4 when the coagulation has started, *e.g.* at 65° C. An addition of calcium carbonate to the extract before heating seems to stabilize the pH to some extent and to simplify the separation of the coagulum. The separation is best made if the solution is allowed to stand for some minutes after the heating is finished, so that the precipitate settles and the clear solution can be siphoned off.

Precipitating the amylase with tannic acid

The purification of malt α-amylase with tannic acid seemed to be worth while studying as the amylase is precipitated out of malt extracts with even very small quantities of the reagent and can be obtained as a water soluble powder if the precipitate is washed with a suitable organic solvent (2, 3), filtered off and dried. As the aim of this part of the present investigation is to find out how best to purify the amylase with tannic acid, experiments have been carried out in the precipitation of the amylase at various pH, the composition of fractionated precipitations and the treatment of the amylase with acetone.

Precipitation at various pH

Malt extract No. 4. 160 g malt meal were extracted for 1 hour with 600 ml 0.01 N $CaCl_2$ solution containing 0.01 % $KBrO_3$. After centrifuging the residue was extracted with another 400 ml and centrifuged. The supernatants were heated to 73° C after adjustment of the pH and filtered. The solution was divided into three parts, which were acidified to pH 5.65, 5.05 and 4.50, respectively. Samples of 50 ml were precipitated at room temperature with various amounts of a 5 % tannic acid solution and the pH adjusted if necessary. After ten minutes the precipitates were centrifuged off and washed once with a solution containing 20 ml 0.3 M phosphate buffer pH 5.5, 2 ml 4 N NaCl and 5 ml 5 % tannic acid solution in 1000 ml water. The centrifuged precipitates were then washed twice with 25 ml acetone and after centrifuging stirred with 1 ml acetone and dissolved in 49 ml 0.4 % calcium acetate solution and centrifuged. The activity and the nitrogen content of the supernatants were assayed. The results are given in Table 3.

Table 3

Fractionated precipitation of a heated malt extract with tannic acid at pH 4.50, 5.05 and 5.65. The activity is given in mg starch/min · ml and the purity in mg starch/min · mg N.

Tannic acid mg	pH = 4.50		pH = 5.05		pH = 5.65	
	Activity	Purity	Activity	Purity	Activity	Purity
(malt extract)	31		66	78	75	85
20	30	260	58	470	70	510
10	17	230	41	560	52	
5	5	110	14	330	19	230

An experiment on the fractionated precipitation of a similar malt extract at pH 6.5 was made in the same manner. The results are given in Table 4.

Table 4

Fractionated precipitation of a heated malt extract with tannic acid at pH 6.5.

Tannic acid mg	Activity	Purity
(malt extract)	48	55
30	40	380
20	38	410
5	33	300

These experiments indicate that the various components of the heated malt extract are precipitated with different ease by the tannic acid, and with increasing amounts of tannic acid other proteins are precipitated both before and after the main part of the amylase is precipitated. The experiments also indicate that the precipitation should not be carried out at a pH value less than 5.

Some further experiments were carried out to show at what pH it is best to precipitate malt α-amylase with tannic acid. Four dry preparations were made up after precipitation at various pH. Some of the procedures employed thereby will be discussed in later sections of this paper.

Preparations No. 33 a–d. 7 kg coarsely crushed malt were stirred with 42 liters of the salt solution recommended. After half an hour the extract was strained and after the malt meal had settled for two hours, the solution was siphoned off. It is suitable to let the solution enter the siphon through a funnel, turned upside down, and connected to the siphon by a rubber tube. After addition of calcium chloride to 0.02 N, and calcium carbonate, the solution was heated to 70° C at pH 6.5 for about 10 minutes. The precipitate soon deposited and the clear solution was siphoned off. When the solution had cooled, some impurities were precipitated with 0.04 g tannic acid per litre, and after some hours the clear solution was siphoned off. The activity of the ex-

Table 5

Amylase preparations made by precipitation with tannic acid at various pH. Assays of 0.5 % solutions.

No.	33 a	33 b	33 c	33 d
pH	5.5	6.5	7.5	8.5
Yield, g	2.1	2.2	1.6	1.8
Activity mg starch/min · ml	228 228	254 234	102 102	15.6 14.6
Nitrogen mg N/ml	0.52 0.52	0.53	0.35 0.36	0.14[1] 0.14

[1] This preparation was not washed with ether before suspension in ether and filtering. It was much darker than the other preparations and had not the same powdery consistency.

tract was 33 mg starch/min · ml after the first precipitation with tannic acid. The solution was divided into 4 equal parts of 6 litres. 1.5 g tannic acid was added to each of them and the pH of the mixture adjusted to 5.5, 6.5, 7.5, and 8.5 respectively. The two slightly alkaline extracts

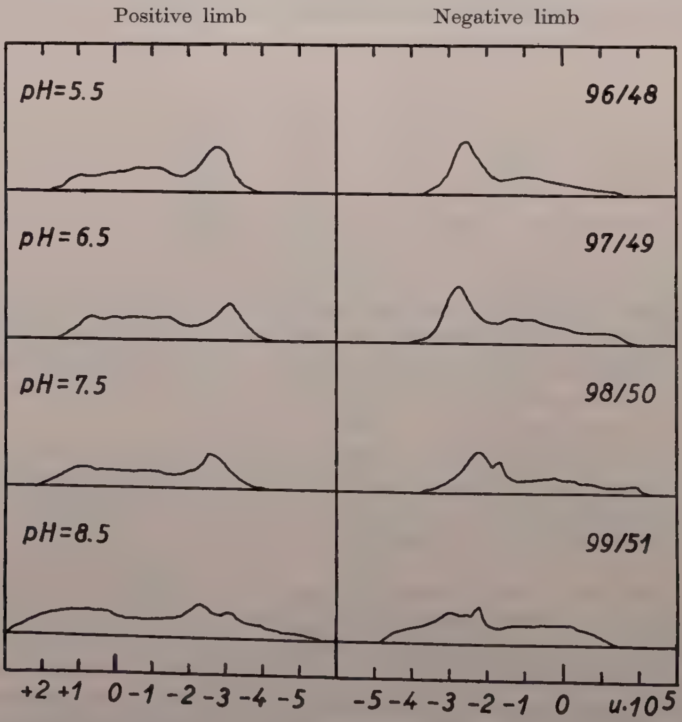

Fig. 2. Electrophoreses at pH 6.25 of amylase preparations precipitated with tannic acid at pH 5.5, 6.5, 7.5, and 8.5 respectively. The preparation at pH 8.5 was carried on for only half the normal time as the concentration was rather low. The electrophoresis no. is given to the right.

assumed a dark colour, and the dry preparations later obtained from them were also rather dark. After the precipitation had deposited for about 2 hours, most of the supernatants were siphoned off and the remainders centrifuged. The precipitates were then suspended in water containing some sodium chloride, calcium chloride and tannic acid, and the pH was adjusted to 5.5. After centrifuging, the precipitates were extracted with 500 ml of a cooled (about 0° C) mixture of 2 parts of purified (22) acetone and 1 part of water and centrifuged. The remaining protein was washed with acetone, centrifuged, washed with ether, centrifuged, suspended in ether, filtered off by suction and dried in vacuo. 0.5 per cent solutions of the powder thus obtained were prepared and the activity and the nitrogen content assayed. The results are given in Table 5.

Electrophoreses were carried out on solutions of these preparations in the usual way in an apparatus with the PHILPOT-SVENSSON optical system (23). The protein content of the solutions was about 2 %. For the buffer — of pH 6.25 — was used a solution containing phosphates of ionic strength $\mu = 0.1$, sodium chloride of $\mu = 0.09$ and calcium chloride of $\mu = 0.01$. The results are given in Fig. 2.

The photographs of the electrophoreses were normally taken after a sufficiently long period for a component with the mobility $u \cdot 10^5 = 1$ to have moved 0.67 cm in the tube, corresponding to 0.5 cm on the plate. This period — about 4 hours — was calculated in each experiment.

The iso-electric points of the various proteins in a purified amylase preparation

A preliminary assay of the iso-electric points of the various proteins in amylase preparations, which have been purified by fractionated precipitation with tannic acid, was obtained by electrophoreses at various pH of solutions of a dry preparation made in the following way.

Preparation No. 11. 4 litres heated malt extract were prepared from 1 kg malt in the way described. A solution containing 0.13 g tannic acid was added. The precipitation was centrifuged off and discarded. A solution containing 2.1 g tannic acid was now added and the pH adjusted to 5.4 with hydrochloric acid. The precipitation was centrifuged off, washed as described, and extracted twice with about 1 liter acetone, suspended in ether, filtered off and dried in vacuo. 4.06 g dry preparation were obtained. The activity of the heated extract before and after the first precipitation was 50 and 40 mg starch/min · ml, respectively. The activity of the dry preparation was 25 500 mg starch/min · g. 0.155 g dry preparation dissolved in 70.7 ml 0.4 % calcium acetate solution had an activity of 59 mg starch/min · ml and total nitrogen content of 0.195 mg N/ml. The 'protein' content — determined (24) as the amount of nitrogen precipitable on addition of 1/10 volume 25 % trichloro-acetic acid — was 0.086 mg N/ml. The purity was 300 mg starch/min · mg N, determined on a centrifuged solution.

Electrophoreses were carried out on solutions of this preparation and on preparation No. 33 to give the iso-electric points of the various proteins present and to show at what pH the best separation is obtained. The protein content of the solutions was about 1.5 %, and the buffer solution had an ionic strength $\mu = 0.2$ as described. The electrophoretic curves are given in Fig. 3.

The electrophoretic patterns are rather vague and they enable only a preliminary assay of the iso-electric points of the various proteins. However, we can conclude from the experiments that there is one component with its iso-electric point 4.6,

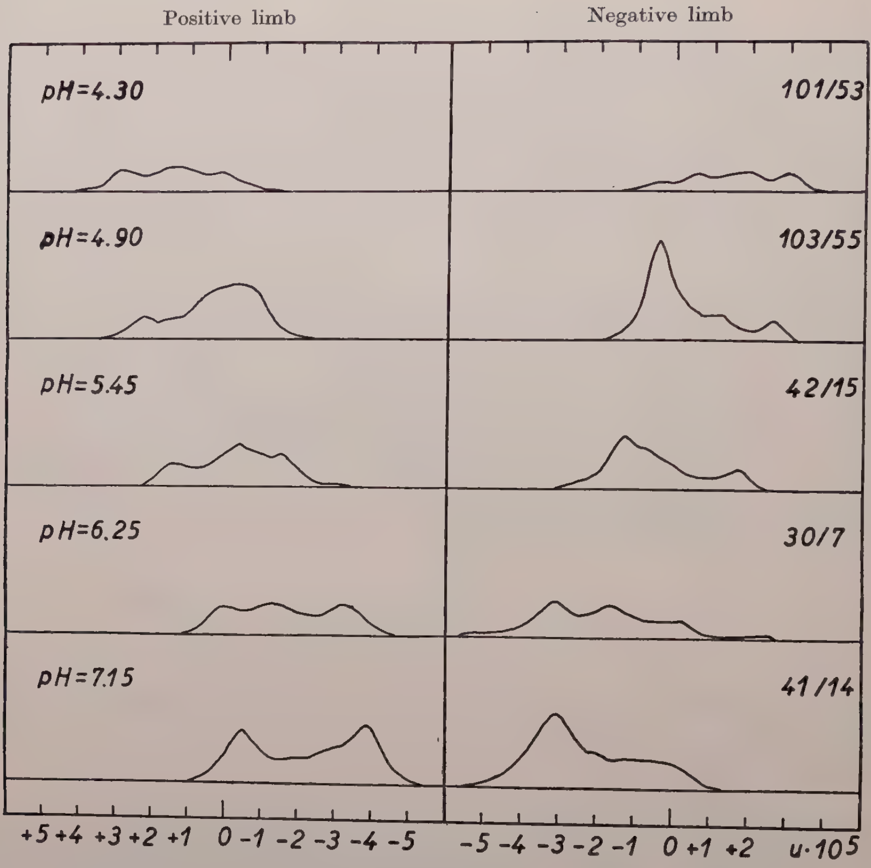

Fig. 3. Electrophoreses at pH 4.30 (prep. No. 33 a), 4.90 (prep. No. 33 b), 5.45, 6.25, and 7.15 (prep. No. 11). The electrophoresis no. is given to the right.

some components with isoelectric points between 5 and 6 and one component with iso-electric point 6.3. The iso-electric points of some malt proteins have been assayed previously. LÜERS and LANDAUER (25), for example, found the value 4.6 for malt albumin, and QUENSEL (9) the values 5.00, 4.90 and 5.70, respectively, for the iso-electric points of the globulins of barley. The iso-electric point of malt α-amylase was determined by DANIELSSON and SANDEGREN (26), who found the value 5.75 and by HULTIN and KRISTIANSSON (27), who found the value 5.9.

Fractionated precipitation with tannic acid

Two experiments were carried out on the fractionated precipitation of malt α-amylase with tannic acid. Malt extracts prepared in the usual way were used. The preparations were analysed for their activity, nitrogen content, 'protein' content and 'proteose and peptone' content.

For ‘protein’ determinations (24) 3 ml of 25 % trichloroacetic acid were added to 30 ml solution. After 1 hour the precipitate was centrifuged, filtered and washed with an equally strong trichloroacetic acid solution, and the nitrogen determined by Kjeldahl’s method. ‘Proteose and peptone’ determinations (24) were made by adding 1 g of acid-washed kaolin to the clear solution from the ‘protein’ assay. The mixture was shaken and centrifuged. The supernatant was discarded and the remainder dried, and the nitrogen content determined as before by Kjeldahl’s method.

Malt extract No. 12. The malt extract was made as follows: 960 g malt meal were extracted with 3 700 ml of the solution recommended. After centrifuging, the remainder was again extracted with 2 400 ml of the solution and centrifuged. The supernatants were collected and $CaCl_2$ added to 0.02 N. The extract was heated to 72° C for 10 minutes at pH 6.5, filtered and cooled.

The fractionated precipitation was made in 11 centrifuge tubes each containing 400 ml solution. A tannic acid solution and 0.5 N hydrochloric acid was added for each precipitation, whereupon the precipitate was centrifuged off. The supernatant was again precipitated. The precipitate was washed with water, containing per litre 250 mg tannic acid and small amounts of calcium chloride, sodium chloride and phosphates. The pH was adjusted to about 5.5. The precipitates were centrifuged, extracted with acetone, suspended in ether, filtered off on a Büchner funnel and dried. The amount of tannic acid and hydrochloric acid added at each precipitation, and the results of analyses of 0.2 % solutions of the preparations thus obtained are given in Table 6.

Table 6

Fractionated precipitation of malt extract No. 12 with tannic acid and analyses of two different 0.2 % solutions of the preparations.

	Original solution	Fraction No.			
		1	2	3	4
Tannic acid, mg/litre		100	250	250	500
0.5 N HCl, ml/litre		0	5	5	2½
pH		6.5	6.0	5.5	4.8
Yield, g		1.57	2.76	1.57	0.93
Activity, mg starch/min · ml	54 54	46 42	79 76	12 12	2.4 2.3
Nitrogen, mg/ml	1.08 1.07	0.203 0.198	0.211 0.204	0.214 0.225	0.214 0.211
‘Protein’, mg N/ml	0.147 0.148	0.112	0.073 0.082	0.059	0.042
‘Proteose and peptone’, mg N/ml	0.181 0.195	0.062	0.072 0.073	0.065	0.062
Purity, mg starch/min · mg N	50	230 210	370 370	56 54	11 11
Amylase yield, mg starch/min.	240 000	33 500	108 000	9 600	1 100

Electrophoreses were carried out as described at pH 6.25 on about 1.5 % solutions of the various fractions in a buffer solution of ionic strenght $\mu = 0.2$. The results are given in Fig. 4 and Table 7.

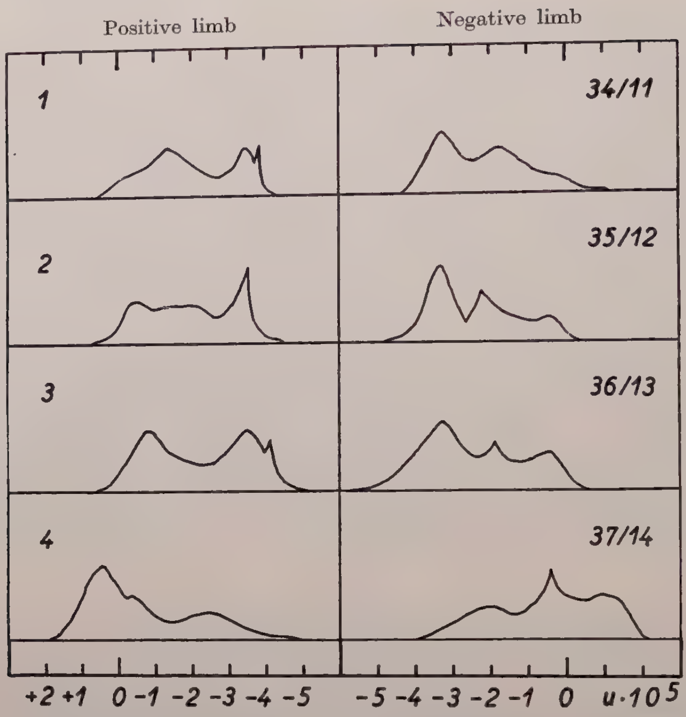

Fig. 4. Electrophoreses at pH 6.25 of amylase preparations obtained by fractionated precipitation of extract No. 12 with tannic acid. The fraction no. is given to the left and the electrophoresis no. to the right.

Table 7

Electrophoretic analyses of amylase preparations obtained by fractionated precipitation with tannic acid. The calculations were made according to the principles suggested by WIEDEMANN (28).

1		2		3		4	
$u \cdot 10^5$	%	$u \cdot 10^5$	%	$u \cdot 10^5$	%	$u \cdot 10^5$	%
Positive limb							
						+0.6	45
−0.5	18	−0.5	26	−0.8	30	−0.4	19
−1.5	31	−1.2	19	−1.5	15	−1.1	9
−2.0	20	−2.1	23	−2.4	12	−2.4	21
−3.4	31	−3.3	41	−3.4	31	−3.3	6
				−4.1	12		
Negative limb							
						+0.9	33
−0.5	14	−0.5	12	−0.4	18	−0.4	25
−1.5	25	−1.2	13	−1.4	10	−1.0	12
−2.1	15	−2.1	26	−1.9	19	−2.1	23
−3.3	46	−3.3	49	−3.3	41	−3.1	7
				−4.3	12		

Another experiment was carried out on the fractionated precipitation with tannic acid, using a similar malt extract (No. 16) and a procedure similar to that of the previous experiment. The results are given in Table 8. The middle fraction was divided into two parts, which were treated separately.

Table 8

Fractionated precipitation of malt extract No. 16 with tannic acid and analyses of the two different 0.2 % solutions of the preparations.

Fraction No.	1	2		3
Tannic acid, mg/l. 0.5 N HCl, ml/l.	40 0	250 5		125 5
Yield, g	0.87	1.50	1.45	1.00
Activity, mg starch/min · ml	32 31	91 90	83 79	37 37
Nitrogen, mg N/ml	0.140 0.152	0.215 0.211	0.199 0.207	0.242 0.231
'Protein', mg N/ml	0.088 0.086	0.090 0.092	0.082 0.083	0.067 0.068
'Proteose and peptone', mg N/ml	0.055 —	0.060 0.068	0.075 0.080	0.069 0.071
Purity, mg starch/min · mg N	230 210	420 420	420 380	150 160
Amylase yield, mg starch/min	14 000	67 000	59 000	18 000

Table 9 gives a list of the heated malt extracts and preparations for which not only the amylase activity and nitrogen content was assayed but also the content of protein precipitable with trichloroacetic acid.

Comparing the results from the malt extracts and from the purified preparations we find that the amylase purity calculated on a 'protein' nitrogen basis varies much less than the purity calculated on a nitrogen basis.

It is evident from these experiments that a considerable purification of the α-amylase can be obtained by fractionated precipitation with tannic acid. If the fractions are suitably chosen, a purification of about 8 times is achieved. Usually some 70 or 80 % of the amylase can be recovered, but if the first fraction is discarded and the remaining amylase not completely precipitated for the main fraction, the yield is somewhat lower. The first fraction is rather dark, and much light absorbing matter is removed with it. This was especially noticed in electrophoretic experiments where a strong light absorbtion is troublesome.

The analyses with trichloroacetic acid and caolin do not give much more information of value, and only indicate that the malt proteins, which are most easily precipitated with tannic acid, are also most easily precipitated with trichloroacetic acid.

The electrophoreses on the various fractions indicate that the various proteins in malt extract are precipitated with different ease with tannic acid and that the frac-

Table 9

Purity of various extracts and preparations.

	$\frac{\text{mg starch}}{\text{min} \cdot \text{mg N}}$	$\frac{\text{mg starch}}{\text{min} \cdot \text{mg protein N}}$
Preparation No. 10	270	625
Extract No. 11	58	385
Preparation No. 11	300	690
Extract No. 12	50	370
Preparation No. 12		
Fraction No. 1	230	390
Fraction No. 2	370	1 000
Fraction No. 3	56	205
Fraction No. 4	11	260
Preparation No. 16		
Fraction No. 1	220	370
Fraction No. 2	420	1 000
Fraction No. 3	155	550
Preparation No. 18	310	760
Extract No. 19	60	600
Extract No. 20	60	600
Preparation No. 22	250	700
Preparation No. 23	375	780

tion containing the main part of the amylase, contains several proteins with perceptibly different mobilities and iso-electric points. As a protein is normally least soluble at its iso-electric point, this simplifies the separation of malt proteins, and outstanding results from such attempts have been published recently by Schwimmer and Balls (29).

Purification of the amylase with acetone

The solubility of the amylase in mixtures of acetone and water

Acetone and various alcohols have been used for the purification of amylases (30). In the present investigation acetone has been employed for extracting tannic acid from amylase preparations and for precipitating amylase, in both cases for making dry preparations of the enzyme. It is therefore of value to know the solubility of amylase in mixtures of acetone and water, and the following experiments were carried out for this purpose.

Preparation No. 28. An amylase solution — obtained by heating a malt extract, precipitating impurities with lead nitrate, fractionated precipitation with tannic acid and with ammonium sulphate and dialysis against 0.0001 N $CaCl_2$ solution — had a purity of 1 200 mg starch/min · mg N. Calcium acetate was added to about 0.005 N and the pH adjusted to 5.9. Samples of 5 ml were cooled to about 4° C and various amounts of cooled acetone added. After about 2 hours the samples were centrifuged and the activity of the amylase in the supernatants assayed. The activity was calculated on the basis of the amylase content in 1 ml of the mixture. The results are given in Fig. 5. For some of the mixtures made up the nitrogen content of the supernatant was also measured. The results are given in Table 10.

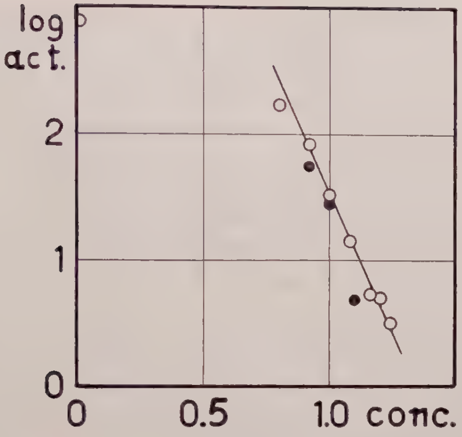

Fig. 5. The solubility of malt α-amylase in mixtures of acetone and water, calculated as mg starch min · ml mixture. The acetone concentration is given as ml acetone added per milliliter water. Rings: normal amylase. Dots: amylase preparation No. 30 with above average solubility in ammonium sulphate solutions.

Table 10

Precipitation of a purified amylase solution with acetone.

Acetone ml/ml water	Activity mg starch/min · ml	Nitrogen mg N/ml
0	780	0.58
1.08	14	0.15
1.20	5.0	0.12
1.24	3.2	0.11

A subsequent paper in this series will report some experiments with preparations, whose amylase showed much higher solubility in ammonium sulphate than is normal, probably as a result of the action of proteolytic enzymes. The amylase of these preparations had almost the same solubility in mixtures of water and acetone as normal amylase preparations. This is also shown in Fig. 5.

The water content in acetone, used for the extraction of tannic acid

The precipitate obtained on addition of tannic acid to a malt extract was treated with dry pure acetone in most experiments in this investigation. In the first attempts at purifying amylase the precipitation was made at a pH of 4.0–4.5. On stirring the precipitate with acetone, it became stickily flocculent after addition of a few times as much acetone as the volume of the precipitate, and powdery on addition of more acetone. In later experiments, when the precipitation was made at a pH of about 6, the precipitate behaved better during the extraction. Some preliminary experiments showed that the tannic acid in these precipitates could be extracted without difficulty with mixtures of acetone and water of very varying strengths. However, the losses of amylase were very great, if mixtures were used containing less than one part of acetone to one part of water. This is quite in agreement with what we can expect from the measurements of the solubility of the amylase given in Fig. 5. As the centrifuged precipitate always contains a good deal of water, a mixture containing two parts of acetone to one part of water was chosen for the extraction. However, a somewhat weaker solution would probably also be suitable.

In extracting the precipitate it is important to be very careful to stir it thoroughly and to add the acetone mixture only in small quantities at first. In most experiments the precipitates were extracted at about 0° C, but treatments at room temperature give nearly as good yields.

If a dry preparation is to be made direct, the residue is suitably centrifuged off and a second extraction made with dry acetone, whereupon the precipitate is again centrifuged off. It is then suitable to suspend it in dry ether, and if the water has not been very well eliminated, it is best to centrifuge and again suspend the powder in ether, and filter it off and dry it in vacuo.

The purification of amylase by precipitating with acetone

A good deal of insoluble matter will remain if we try to dissolve in water a dry preparation made in the way just described or the centrifuged residue from the extractions with acetone (even the second extraction of the tannic acid may then be made with a mixture of acetone and water; dissolving the precipitate is rendered simpler if it is first stirred with a small amount of the mixture). The insoluble matter is best centrifuged off at a pH of 4.6, where also the solubility of the malt main albumin is least.

If we know the activity of the solution, we can easily calculate from Fig. 5 how much acetone must be added to get a given percentual yield of amylase. In most cases 1.1 volume of acetone can be recommended.

After the precipitate has been centrifuged off, it is suitable to wash it with acetone and with dry ether, to suspend it in ether and filter it off by suction, and to dry it in vacuo.

The production and analyses of two preparations will be reported here.

Preparation No. 31. 1.5 kg malt meal were extracted in the usual way and 8 litres of a heated solution obtained, the activity of which was 35 mg starch/min · ml. A first precipitate with 0.2 g tannic acid was discarded, and the activity of the remaining solution was 33 mg starch/min · ml. The precipitate then obtained with 4 g tannic acid was extracted twice with the mixture of acetone and water and dissolved in 600 ml cooled 0.002 N $CaCl_2$ solution and centrifuged at pH 4.6 (+5° C). After the pH was adjusted to 5.9, 1.1 volume of cooled acetone was added and the precipitate centrifuged after half an hour and treated in the way recommended. 1,53 g dry preparation were obtained, the activity of which was 94 000 mg starch/min · g and the purity 980 mg starch/min · mg N.

For another preparation of amylase (No. 32) 7 kg of coarsely crushed malt were treated with 42 litres of the salt solution, and 19 litres of extract were siphoned off. Treatment with another 19 litres gave a second extract of 19 litres. After heating, 17 $^1/_2$ litres remained of each extract, and a precipitate obtained with 0.04 and 0.02 g tannic acid per litre extract, respectively, was discarded. After siphonong, 32 litres of mixed extract with an activity of 32 mg starch/min · ml remained. The amount of amylase was thus 1 000 000 mg starch/min.

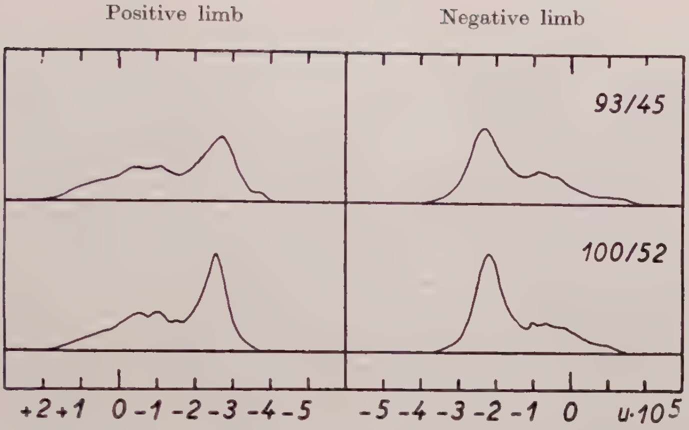

Fig. 6. Electrophoreses of the two amylase preparations purified as recommended with tannic acid and acetone. Upper curve: preparation No. 31. Lower curve: preparation No. 32.

Two similar extractions with a somewhat better yield were also made (after straining the meal was allowed to seattle and 31 litres were siphoned off as the first extract). The amylase was precipitated at room temperature as usual with 7.5, 9.5 and 9.5 g tannic acid, respectively.

After two treatments with the mixture of acetone and water, the precipitate was dissolved in 8 litres of a 0.002 N $CaCl_2$ solution. The activity was 290 mg starch/ml and the nitrogen content 0.48 mg N/ml. Thus the purity was 590 mg starch/min · mg N and the total amylase content 2 300 000 mg starch/min. The precipitate centrifuged off at pH 4.6 contained 52 000 mg starch/min of amylase (the precipitate was suspended in 180 ml water and the activity measured).

15.24 g dry preparation were obtained. A 2 % solution had an activity of 2150 mg starch/min · ml and nitrogen content 2.83 mg N/ml. Thus the purity was 760 mg starch/min · mg N and the total amylase yield 1 640 000 mg starch/min. The percentual yield from the extracts was 46 %.

The last amylase preparation was completely soluble in water after a slight increase in the pH. Electrophoreses were run on solutions of these preparations at pH 6.25 and $\mu = 0.2$. The results are given in Fig. 6.

The stability of the purified amylase

Only some preliminary experiments have been made on the stability of the purified amylase.

A 0.5 % solution of the preparation last described was dialyzed against distilled water for two days, whereby globulins were precipitated. After centrifuging the supernatant was made 0.02 N as regards $CaCl_2$ and the pH adjusted to 6.5. The activity measurements are given in Table 11.

Table 11

Stability of a purified amylase preparation at pH 6.5 in a 0.02 N $CaCl_2$ solution.

Time hours	0	3	21	45	69	99	192
Activity mg starch/min · ml	420	405	405	335	300	310	305

The purified amylase seems to have good stability.

SUMMARY

If a 0.01 % solution of potassium bromate, containing a suitable quantity, 0.002 N, calcium chloride, is used for the extraction of α-amylase from malt, the proteolytic enzymes are strongly inhibited. The heat inactivation at about 70° of other enzymes and the simultaneous coagulation of some proteins is best made at a pH of 6.4 after the addition of calcium salts to about 0.02 N. The bromate has no influence on the activity or stability of the amylase.

The precipitation with tannic acid is best made at a pH of about 6. A first small precipitate, containing impurities, should be discarded. After centrifuging the main precipitate, the tannic acid is best extracted twice with a mixture of acetone and water (2 + 1 or somewhat weaker). It is advisable to dissolve the remainder in water, preferably containing some calcium salts. The insoluble matter is best centrifuged off at a pH of 4.6, and after the pH has been adjusted to 5.9, the amylase can be precipitated with 1.1 volumes of acetone. For making a dry preparation, the precipitate is best washed with dry acetone and dry ether, filtered off by suction and dried in vacuo. A purification of about 15 times calculated on the nitrogen content is achieved by the treatment with tannic acid and acetone.

Electrophoretic investigations indicate that the various proteins in a heated malt extract are precipitated with varied ease and that there are proteins with the iso-electric points 4.5, 5–6 (several proteins) and 6.3 in the purified amylase preparations. About half the amount of protein in the best amylase preparations is albumin with iso-electric point 4.6. The preparations also contain globulins.

The purified amylase has good stability in solutions containing calcium salts.

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Investigations on malt amylase

VII. First report on the purification of α-amylase with ammonium sulphate and dialysis

By ESKIL HULTIN

With 9 figures in the text

Ammonium sulphate is one of the most commonly used reagents for the separation and purification of proteins. During the last few years it has been used successfully for the purification of various α-amylases, and among the papers on the subject may be mentioned those by FISCHER (1), SCHWIMMER (2), DANIELSSON and SANDEGREN (3), and SCHWIMMER and BALLS (4). The purpose of the present investigation — whose first results are given in this paper — is to find out the best conditions for the purification of malt α-amylase that has first been partly purified with tannic acid and acetone (5).

The specific property solubility test

An interesting analysis of the use of ammonium sulphate for the purification of enzymes has been made by FALCONER and TAYLOR (6), who suggest a specific property solubility test. They consider the purification of an enzyme which occurs together with two other proteins, one of which is more soluble and the other less soluble in ammonium sulphate than the enzyme. The enzyme may, however, occur together with a protein that is less soluble than the enzyme in weak ammonium sulphate solutions and more soluble than the enzyme in strong ammonium sulphate solutions. In such cases both the enzyme and the protein are precipitated at the same time. This is shown by the following experiment.

3 g of amylase preparation No. 28 the purity of which was 400 mg starch/min · mg N, were dissolved in 100 ml of half saturated calcium sulphate solution. After centrifugation, the solution was cooled and 23 g ammonium sulphate were added. After two hours the precipitate was centrifuged off and dissolved in 100 ml of half saturated calcium sulphate solution and dialyzed against a 0.0001 N calcium chloride solution for two days, whereby globulins were precipitated. The mixture was centrifuged, and 0.3 ml 2 N calcium chloride and 1 ml of acetate buffer (equal parts of 1 N HAc and 1 N NaAc) were added to the supernatant (pH = 4.9). Samples of 10 ml were made up and various amounts of ammonium sulphate (0 to 2.13 g) were added. The samples were left for some hours in the refrigerating room at +4° C and centrifuged. The activity of the supernatant was measured colorimetrically (7). The protein content of the supernatant was assayed by ROCHE and MARQUET's (8) method. No volume corrections were made for the ammonium sulphate. The results are given in Table 1 and Fig. 1.

Table 1.

Precipitating a purified amylase solution with ammonium sulphate at + 4° C and pH = 4.9.

$(NH_4)_2SO_4$ g/ml water	Activity mg starch/min · ml	Protein mg N/ml
0.000	470	0.81
0.050	440	0.66
0.100	430	0.44
0.125	250	0.35
0.138	230	0.37
0.150	150	0.29
0.162	155	0.26
0.175	69	0.21
0.188	32	0.16
0.200	25	0.14
0.213	13	0.12

It is not possible to calculate the maximum amylase activity per mg N from such a graph as the one in Fig. 1. The concentration of protein which is more soluble than the enzyme in strong ammonium sulphate solutions must first be reduced — by purification of the enzyme or possibly by dilution of the solution or by choosing a more suitable pH — so that the main part of the enzyme is precipitated before the solution becomes saturated with the protein in question, on addition of increasing quantities of ammonium sulphate.

If, for a first approximation — which will be discussed in a subsequent section — we assume that the activity of the pure amylase is 4000 mg starch/min · mg N, and substract the amylase protein from the total protein (Table 1) we get a nitrogen value for the impurities. In Fig. 2 the logarithms of the protein nitrogen for the amylase and for the impurities are plotted against the amount of ammonium sulphate added per ml.

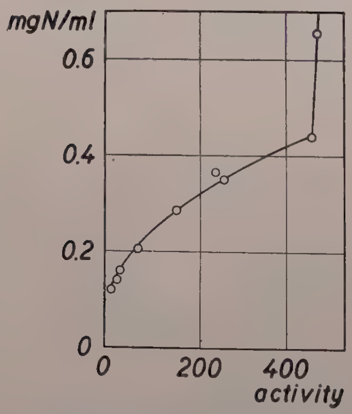

Fig. 1. Specific property solubility test of a purified solution of amylase preparation No. 28 at pH 4.9.

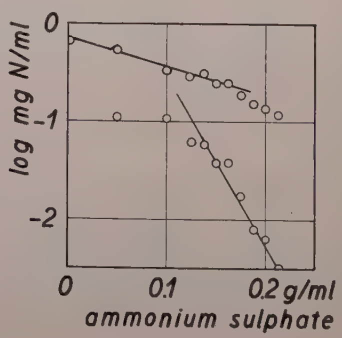

Fig. 2. The logarithms of the protein nitrogen for the amylase (lower curve) and for the impurities (upper curve) plotted against the ammonium sulphate concentration, assuming the maximum amylase activity = 4000 mg starch/min · mg N. Calculations from Table 1.

The slopes of the two curves are so different that we may expect to get a considerable purification of the amylase by repeated fractioning with ammonium sulphate.

Another specific property solubility test was made at pH = 6.5, using preparation No. 32, which has been described in a previous paper (5). In this experiment the pH and the purity of the preparation were probably more suitable for the test, but the solubility properties of the amylase were not so good as in the first test, as may be seen from Fig. 6. The results of the measurements are given in Fig. 3. Even in this experiment another protein is precipitated at the same time as the amylase. The main slope of the line gives the maximum activity 1430 mg starch/min · mg N for the precipitate, which is obviously much less than the maximum activity of the amylase.

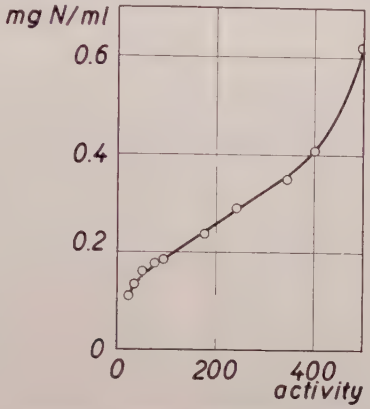

Fig. 3. Specific property solubility test of amylase preparation No. 32 at pH 6.5.

The solubility of malt α-amylase in ammonium sulphate solutions

It has been reported by SCHWIMMER (2) that the solubility of the amylase in ammonium sulphate solutions is increased and rendered more similar to that of the other proteins in a malt extract if the extract is allowed to stand at room temperature for some days before heating. HOLMBERGH (9) has found in some experiments that the amylase was not precipitated even if the solution was saturated with ammonium sulphate.

Amylase preparations, made from freshly milled malt from the same batch in the way described previously (5), always showed the same solubility in ammonium sulphate solutions, irrespective of the age of the malt. For some preparations crushed malt was used, and then the properties of the amylase changed rather with the age of the malt.

Preparation No. 28, which has been described previously (5), was made a few days after the malt was crushed. The preparation is also mentioned in a subsequent section.

Preparation No. 29. The malt for this preparation — 11 kg — was finely crushed about 2 weeks before it was used. The precipitation with 18 g of tannic acid was obtained in the usual way, washed with 1 litre of distilled water, and freeze dried. 47.3 g of the compound were ob-

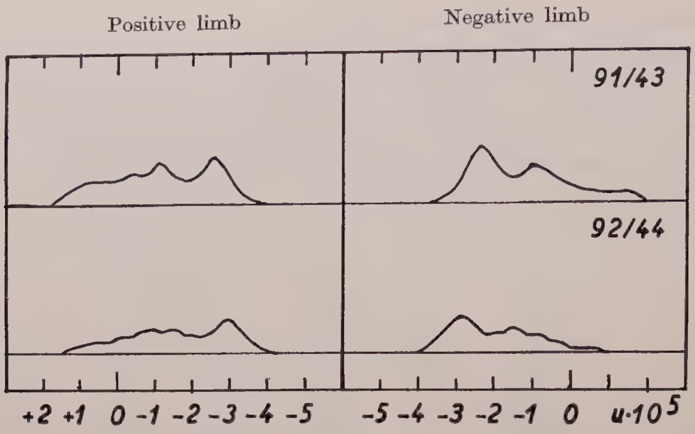

Fig. 4. Electrophoreses at pH 6.25 and μ = 0.2 of preparation No. 30. Upper curve: the original preparation. Lower curve: a reprecipitated preparation.

tained. Amylase preparations were made in the usual way by extracting the tannic acid with a mixture of acetone and water, whereupon the remainder was washed with acetone and with ether, filtered off and dried. The losses of amylase were greater than in other preparations, and there appeared to be no advantage in drying the tannic acid precipitate. The purity of the preparation obtained was 19 000 mg starch/min · g dry substance and 320 mg starch/min · mg N determined on a centrifuged solution.

Electrophoreses carried out on this preparation are reported later on and given in Fig. 8.

For solubility determinations a solution of the activity 400 mg starch/min · ml and nitrogen content 1.50 mg N/ml was used.

Preparation No. 30. This preparation was made about 4 weeks after the malt was crushed. 7 kg meal were extracted with 42 litres $CaCl_2$ and $KBrO_3$ solution, and 25 litres extract siphoned off. A second extraction of 26 litres was also obtained. After heating to 70° the activities were 48 and 21 ml starch/min · ml respectively. 0.04 and 0.02 g of tannic acid per litre respectively were added and the supernatant siphoned off. The activities were 44 and 21 mg starch/min · ml respectively. The extracts were mixed and precipitated with 9 g of tannic acid at pH 5.1. The precipitate was allowed to settle for two hours. Most of the supernatant was siphoned off. The precipitate was then centrifuged and extracted twice with a mixture of 2 litres of acetone and 1 litre of water. After centrifugation the precipitate was dissolved in 800 ml half saturated calcium sulphate solution and the insoluble matter centrifuged off. The activity was 810 mg starch/min · ml and the nitrogen content 1.62 mg N/ml. Hence the purity was 500 mg starch/min · mg N and the yield 650 000 mg starch/min, corresponding to 53 %.

0.20 g ammonium sulphate was added per ml and pH adjusted to 5.9. The precipitate was centrifuged off at about +5° C and contained 165 000 mg starch/ml of amylase. The activity of the supernatant, 740 ml, was 600 mg starch/min · ml. Ammonium sulphate was added to increase the concentration to 0.27 mg/ml, but only a small precipitate, containing 33 000 mg starch/min of amylase was obtained. As the amylase did not behave normally on addition of ammonium sulphate, the supernatant was treated in the following way. It was first dialyzed against tap water for 18 hours. Some experiments showed (5) that it could be precipitated with acetone in the normal way, and a dry preparation was made up. Thereby 3.59 g dry preparation were obtained, the purity of which was 1250 mg starch/min · mg N. The percentage yield from this acetone precipitation was 81 %.

Electrophoreses were carried out on this preparation and on a preparation obtained by reprecipitating it with 1.1 vol. acetone from a 0.3 % solution. The results are given in Fig. 4.

For solubility determinations a 0.2 % solution of this preparation was used.

Preparation No. 35. This preparation was made from the same lot of crushed malt as preparations No. 28, 29, and 30. Its age (after crushing) was about 3 months. The humidity of the meal was 8 %. For the last two months it was stored in a dryer place than before. The preparation was made as recommended. The purity was 830 mg starch/min · mg N. For solubility determinations a 0.5 % solution was used.

The solubility of the various preparations was determined at pH 5.9 — in the case of preparation No. 28 at pH 4.9 — and at about + 4° C. Various amounts of ammonium sulphate were added to samples of 10 ml and after about 2 hours the samples were centrifuged. The results are given in Fig. 5.

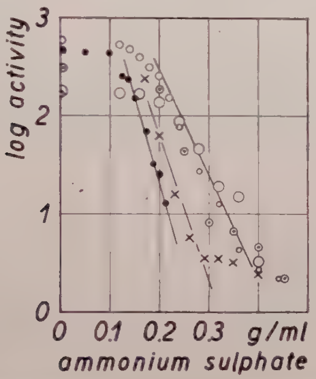

Fig. 5. The solubility at + 4° C of amylase preparations made at various intervals after the malt was crushed. Dots: preparation No. 28 at pH = 4.9. Crosses: preparation No. 29 at pH = 5.9. Rings of different size: preparation No. 30, various experiments at pH = 5.9. Rings with a dot: preparation No. 35 at pH 5.9.

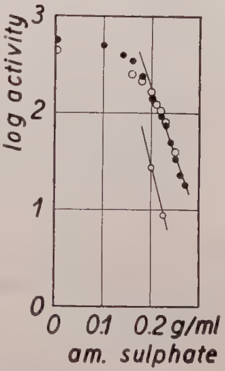

Fig. 6. The solubility at + 4° C of amylase preparations No. 18 at pH 5.5 (small rings) and No. 32 at pH 5.9 (large rings) and pH 6.5 (dots).

The solubility of amylase preparation No. 32, which has been described previously (5), was determined at pH 5.9 and 6.5, using 0.4–0.5 % solutions. The results are given in Fig. 6. The solubility of preparation No. 18 is also given.

From these experiments we conclude that various amylase preparations may show rather different solubility in ammonium sulphate solutions, and that the properties of the α-amylase in milled malt can be altered by time. The experiments also indicate that the amylase can be partly precipitated together with the impurities even before sufficient ammonium sulphate has been added for the solution to be reasonably assumed to be saturated with the amylase.

In the experiments reported here the amylase content of a saturated amylase solution is reduced to half on increasing the ammonium sulphate concentration with 0.017 g/ml water in the case of preparations No. 18 and 28, whereas 0.028 g/ml are required for a corresponding precipitation of preparation No. 30. It is obvious that the purification of a protein is facilitated if almost all of it can be precipitated by only a slight increase of the concentration of ammonium sulphate.

It is reasonable to assume that the varying solubility of different amylase preparations is due to the action of proteolytic enzymes and — as a consequence of this — that amylase molecules of different solubility — possibly of different molecular weight — are present at the same time in the solutions. This hypothesis is supported by the results of the solubility measurements at low amylase solubility given in Fig. 5 (preparations No. 29 and 35).

The fractionated precipitation

Some experiments were carried out on the purification of amylase preparations made in the way recommended (5), without, however, fractionated precipitation with acetone.

Preparation No. 18. Activity 310 mg starch/min · mg N. 'Protein' (5) N 41 %. 'Proteose and peptone' (5) N 31 %. 20 ml of a 2 % solution of pH about 5.5 were poured into a centrifuge tube and 1 g ammonium sulphate added. After about half an hour at +4° C the mixture was centrifuged. The precipitate was dissolved in 20 ml 0.4 % calcium acetate solution, and the supernatant poured into another centrifuge tube and another 1 g ammonium sulphate added and so on. The activity and protein nitrogen (8) were measured and the results are given in Table 2.

Table 2.

Fractionated precipitation of amylase preparation No. 18 with ammonium sulphate.

$(NH_4)_2SO_4$ g/ml water	Activity mg starch/min · ml	Protein mg N/ml	Purity mg starch/min · mg N
(original solution)	290	1.21	240
0.00—0.05	5	0.034 0.032	150
0.05—0.10	29	0.132	220
0.10—0.15	96	0.144 0.148	660
0.15—0.20	120	0.154 0.148	800
(supernatant)	28	0.457 0.463	61

A better purification was obtained using preparation No. 28.

2 kg finely crushed malt meal were extracted with 8 $^1/_2$ litres of the $CaCl_2$ and $KBrO_3$ solution, giving 6.3 litres of extract. After heat coagulation 2 g lead nitrate were added and the precipitate centrifuged off. Then a dry preparation was made in the usual way, but without fractionated precipitation with acetone. The purity of the preparation was 400 mg starch/min · mg N.

The precipitate obtained at pH 5.5 from a 2 % solution when the ammonium sulphate concentration was increased from 0.10 to 0.20 g/ml was used for electrophoresis No. 81/33, given in Fig. 7.

A 4 % solution was precipitated with 0.18 mg ammonium sulphate/ml at pH 6.4 and the precipitate dissolved and dialyzed against a 0.0001 N calcium chloride solution. The globulins pre-

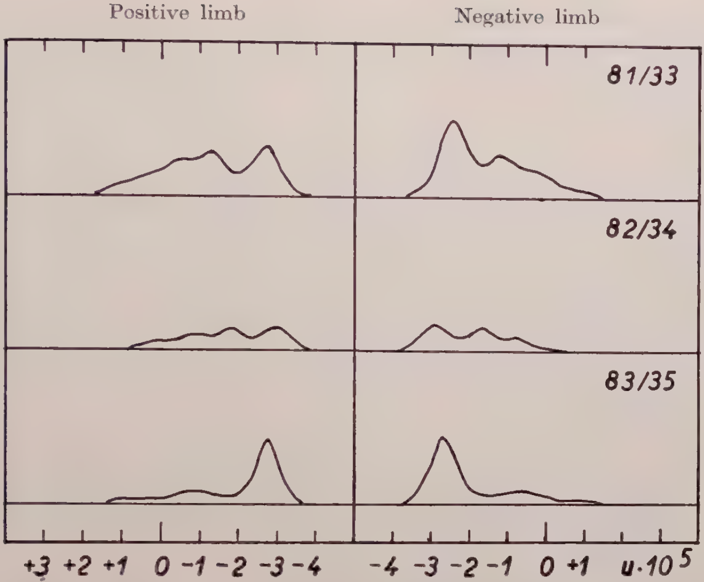

Fig. 7. Electrophoreses of preparation No. 28 at pH = 6.6 and μ = 0.2. Top curve: Fraction between 0.10 and 0.20 g ammonium sulphate/ml. Middle curve: purified amylase fraction. Bottom curve: first fraction.

cipitated thereby were centrifuged off. The purity of the amylase was now 560 mg starch/min · mg N.

The solution contained 0.80 mg protein N/ml. A first precipitate was obtained at pH 4.8 with 0.1 g ammonium sulphate/ml and used for electrophoresis No. 83/35. The main fraction was obtained by increasing the ammonium sulphate content to 0.18 g/ml water. This fraction, which had the purity 1200 mg starch/min · mg N was used for electrophoresis No. 82/34, given in Fig. 7. The composition of the preparation is given in Table 3.

Table 3.

Electrophoretic analysis of the purified preparation No. 28 (electrophoresis 82/34).

Positive limb		Negative limb	
Mobility $u \cdot 10^5$	%	Mobility $u \cdot 10^5$	%
−2.90	45	−2.85	47
−1.75	37	−1.65	36
−0.80	18	−0.80	17

Electrophoreses were carried out on some fractions of preparation No. 29, obtained with ammonium sulphate in the following way.

A 2 % solution of the preparation was precipitated at pH 5.9 and +4° C with 0.18 g ammonium sulphate/ml. The precipitate was dissolved and reprecipitated at pH 4.6 with 0.10 g ammo-

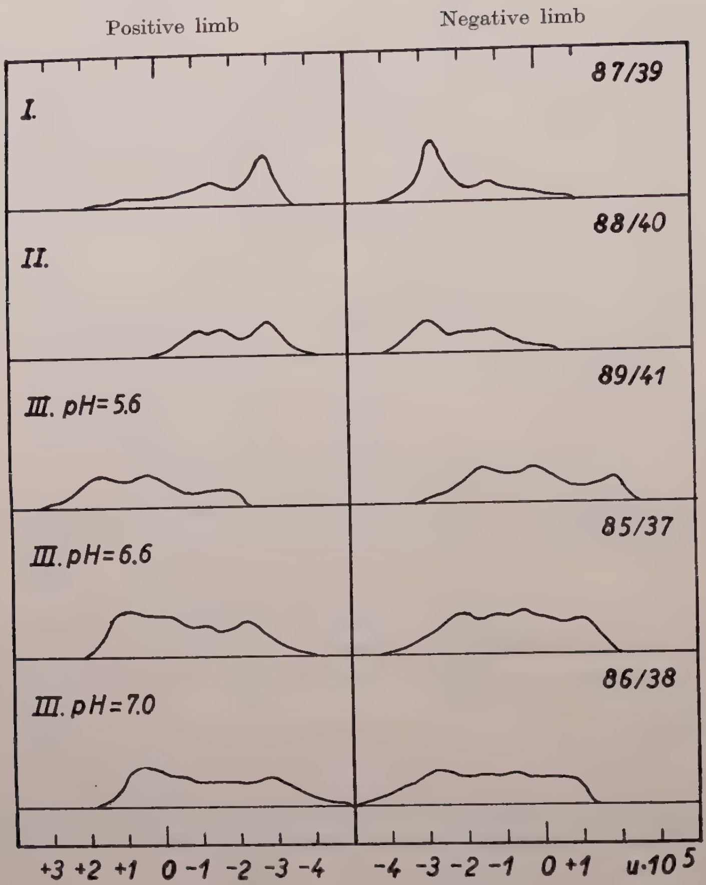

Fig. 8. Electrophoreses on fractions of preparation No. 29. No. 87/39, first precipitate. No. 88/40, second precipitate. Nos. 89/41, 85/37 and 86/38, remaining protein.

nium sulphate per ml. Electrophoresis No. 87/39 was carried out at pH 6.6 and $\mu = 0.2$ on a solution of this preparation (I).

The supernatant was precipitated at pH 6.0 by increasing the ammonium sulphate content to 0.26 g/ml. After reprecipitating twice with 0.22 g ammonium sulphate/ml from solutions containing about 0.5 mg protein N/ml, the fraction (II) was used for electrophoresis No. 88/40 at pH 6.6. The purity was 650 mg starch/min · mg N. A last fraction (III) was obtained by saturating the supernatant from the middle fraction with ammonium sulphate. Using this fraction, electrophoreses No. 89/41, 85/37 and 86/38 were carried out at pH 5.6, 6.6, and 7.0 respectively. The electrophoresis curves are given in Fig. 8.

The albumin with its iso-electric point at about pH = 4.5 can easily be recognized in all the electrophoretic curves, but the first fraction has the highest percentage content of this albumin. The last fraction contains proteins with iso-electric points at about pH = 4.5, 5.6, 6.4, and 7.4.

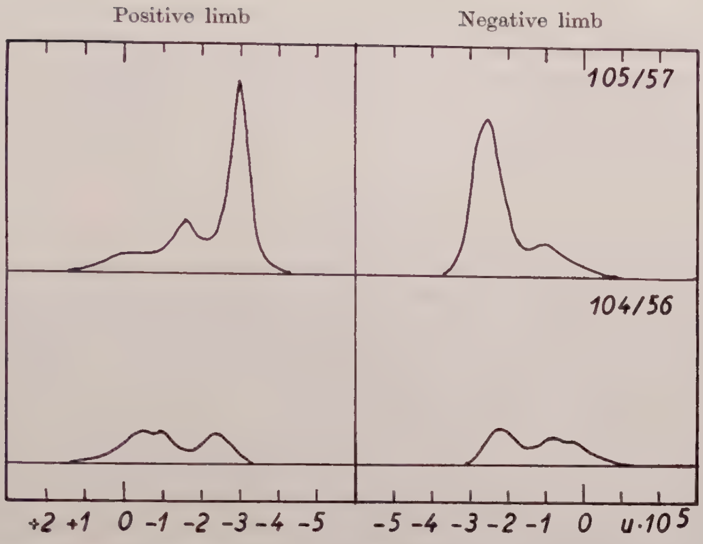

Fig. 9. Electrophoreses at pH 6.25 and $\mu = 0.2$ of the purified preparations No. 32 (upper curve) and 33 (lower curve).

In two preparations the fractionated precipitation with ammonium sulphate and with acetone were combined, and electrophoreses of the preparations were carried out at pH 6.25 and $\mu = 0.2$. The electrophoreses were as usual carried on for about 4 hours. The curves are given in Fig. 9.

Purified preparation of malt extract No. 32. 4 g of preparation No. 32 were dissolved in 800 ml of half saturated calcium sulphate solution and the pH adjusted to 6.5. 0.26 g ammonium sulphate per ml were added and the precipitate dissolved and reprecipitated in the same way. The precipitate was dissolved in 100 ml of half saturated calcium sulphate solution and dialyzed against a 0.0001 N calcium chloride solution. After centrifugation a dry preparation was made in the usual way. The purity of the preparation was 750 mg starch/min · mg N. The yield was 0.57 g.

Purified preparation of malt extract No. 33. The second extract obtained from the meal was precipitated with 4 g tannic acid and extracted with a mixture of acetone and water in the usual way. It was dissolved in 1.4 litres of 0.001 N calcium chloride solution and centrifuged at pH 4.5. The first precipitate obtained at pH 4.6 with 0.12 g ammonium sulphate/ml was discarded. A second precipitate obtained on increasing the ammonium sulphate content of 0.24 g/ml was dissolved in 85 ml half saturated calcium sulphate solution and dialyzed against a 0.0001 N calcium chloride solution. After centrifugation the solution was precipitated with 1.1 volumes of acetone and a dry preparation made in the usual way. The purity of the preparation was 1430 mg starch/min · mg N.

The maximum activity of the amylase

Schwimmer and Balls (4) found that the activity of the pure α-amylase calculated per mg N was about 30 times that of a heated malt extract. The electrophoresis of the purified amylase preparation No. 28 (Fig. 7) shows one protein component with a mobility similar to what we may expect for the amylase. As this component is about 35 % of the total amount of protein, and the sample had an activity of 1200 mg starch/min · mg N, the activity of the pure amylase is not less than 3500 mg starch/min · mg N, which implies a purity of about 60 times that of a heated extract of the malt used in this investigation. Similar calculations from electrophoreses No. 105/57 and 104/56 (Fig. 9) give values of about 3500–4500 mg starch/min · mg N for the maximum amylase activity.

Conclusion

The purification of malt α-amylase with ammonium sulphate is rendered difficult by the fact that the solubility of various amylase preparations differs considerably. It was assumed long ago by OSBORNE (10, 11) that amylase is closely related to albumin, and DANIELSSON and SANDEGREN (3) have recently found that the amylase activity of a preparation increases with increasing purity of the albumin. However, the iso-electric point of malt α-amylase differs considerably from that of common malt albumin, and the solubility of amylase that has not been altered by autolysis also differs considerably from that of common albumin, as it is almost completely precipitated in half saturated ammonium sulphate solution.

The results so far obtained seem to indicate that the following points should be taken into consideration when purifying malt α-amylase with ammonium sulphate. Autolysis should be avoided. At the first fractionation a first precipitate of impurities is best taken at pH about 4.5, where the solubility of the albumin is low. The main fraction is best taken at a pH about 6–7. The amount of ammonium sulphate that should be added for each fraction must first be investigated for each lot of amylase preparation, as the solubility of various preparations may differ considerably. The fractionated precipitation should be made in dilute solutions. In strong solutions a good deal of the amylase follows the precipitate of impurities, although the solution is not reasonably saturated with the amylase. In dilute solutions a great deal of the impurities remain in the solution even when almost all the amylase has been precipitated. In connection with the fractionated precipitation with ammonium sulphate it is suitable to precipitate the globulins by dialysis for a few days in cellophane bags against distilled water or a 0.0001 N calcium chloride solution.

SUMMARY

Albumin with iso-electric point at pH = 4.5 is less soluble than the amylase in weak ammonium sulphate solutions and more soluble than the amylase in strong ammonium sulphate solutions. It can be partly removed from amylase solutions containing this albumin as a main protein constituent by precipitation with ammonium sulphate at pH about 4.5.

The amylase fraction is best obtained from dilute solutions at pH about 6–7, whereby it is necessary first to measure the solubility of the actual lot of amylase in ammonium sulphate solutions, as it varies rather.

If an enzyme solution containing one impurity that is less soluble in weak ammonium sulphate solutions and more soluble in strong solutions than the enzyme, the specific property solubility test cannot be applied until the quantity of this impurity has been so much reduced that the main part of the enzyme is precipitated before the solution becomes saturated with the impurity.

This investigation was assisted financially by *Statens Naturvetenskapliga Forskningsråd*. The electrophoresis apparatus was provided by *Statens Tekniska Forskningsråd*. Professor KARL MYRBÄCK kindly granted me the use of his laboratories. The malt was obtained as a gift from AB Stockholms Bryggerier. The electrophoreses were carried out by fil. kand. INGRID KRISTIANSSON. Miss IRMA SJÖGÅRDH assisted in carrying out the other experiments. The English text was revised by Mrs FRANCINA CAMERON. For all this help I wish to express my cordial thanks.

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Investigations on malt amylase

VIII. On the determination of dissociation constants for the compounds of enzymes and their stabilizing metal ions

By ESKIL HULTIN

For enzymes that are stabilized by certain metal ions we can presume that the actual stability of the enzyme in a solution of the metal salt of given strength and given pH depends on the maximum stability the metal ion can give the enzyme, and the dissociation constant for the compound between the metal ion and the part of the enzyme essential for the maintenance of its activity. It may be that the metal ion can combine with other parts of the enzyme, too, but this article concerns only the dissociation at the active part of the molecule.

Assuming the inactivation of the enzyme to be a reaction of the 1st order, we apply the following notation in a theoretical treatment of the problem.

E_u = the unstable enzyme (the enzyme not combined with the metal ion),
E_s = the stabilized enzyme (the compound between the enzyme and the stabilizing ion),
E = the enzyme (both unstable and stabilized),
M = the metal ion,
K = the dissociation constant of the stabilized enzyme,
k_u = the rate constant for the inactivation of the unstable enzyme,
k_s = the rate constant for the inactivation of the stabilized enzyme,
k = the rate constant when the activity of the metal ion is [M],
t = the time.

Square brackets are used for denoting the concentration of the enzyme and the activity of the metal ion.

If we assume that the following equation is valid for the dissociation of the stabilized enzyme

$$E_s = E_u + M, \tag{1}$$

we have

$$\frac{[\mathrm{E_u}] \cdot [\mathrm{M}]}{[\mathrm{E_s}]} = K \tag{2}$$

and

$$[\mathrm{E_s}] = \frac{[\mathrm{M}]}{K} [\mathrm{E_u}] . \tag{3}$$

As

$$[\mathrm{E}] = [\mathrm{E_u}] + [\mathrm{E_s}] ,$$

we have

$$\mathrm{d}\,[\mathrm{E}] = -\,[\mathrm{E_u}]\, k_{\mathrm{u}}\, \mathrm{d}\, t - [\mathrm{E_s}]\, k_{\mathrm{s}}\, \mathrm{d}\, t \tag{4}$$

$$\mathrm{d}\,[\mathrm{E}] = -\,[\mathrm{E}] \frac{k_{\mathrm{u}}}{1 + \frac{[\mathrm{M}]}{K}} \mathrm{d}\, t - [\mathrm{E}] \frac{\frac{[\mathrm{M}]}{K} k_{\mathrm{s}}}{1 + \frac{[\mathrm{M}]}{K}} \mathrm{d}\, t \tag{5}$$

From this equation we can easily obtain k:

$$k = \frac{k_{\mathrm{u}} + \frac{[\mathrm{M}]}{K} k_{\mathrm{s}}}{1 + \frac{[\mathrm{M}]}{K}} \tag{6}$$

and hence

$$K = \frac{[\mathrm{M}]}{\frac{k_{\mathrm{u}} - k}{k - k_{\mathrm{s}}}} . \tag{7}$$

If the rate constant for the inactivation of the stabilized enzyme is small, the last expression simplifies to

$$K = \frac{[\mathrm{M}]}{\frac{k_{\mathrm{u}}}{k} - 1} , \tag{8}$$

which implies that the constant has the same numerical value as the metal ion activity when the rate constant for the inactivation is half that of the inactivation in the absence of the ion.

Similarly, if we assume the following equation for the dissociation of the stabilized enzyme:

$$[\mathrm{E_s}] + [\mathrm{H^+}] = [\mathrm{E_u}] + [\mathrm{M}] \tag{9}$$

we get

$$K = \frac{[\mathrm{M}]}{[\mathrm{H^+}]} : \frac{k_{\mathrm{u}} - k}{k - k_{\mathrm{s}}} . \tag{10}$$

By carrying out experiments at various pH it should be possible to find out which of equations (1) and (9) is valid for the dissociation of the stabilized enzyme or if some other, similar equation is valid.

It would be of particular interest to prove the validity of these equations for the stabilization of malt α-amylase with calcium ions. There are, however, at present several difficulties to overcome before an accurate assay can be made. The inactivation of the amylase preparations examined hitherto by the present author is only approximately a reaction of the first order (1, 2). As the amylase is stabilized appreciably in a 0.0002 N calcium salt solution (3), it is evident that an accurate assay can only be made if the calcium ion activity can be measured in enzyme solutions with considerably less calcium content. It seems to be impossible to do this with electrodes, using the methods hitherto employed (4).

An attempt to get the order of magnitude of the dissociation constant was made using the results of earlier measurements (5) of the inactivation of enzyme solutions strongly diluted with calcium chloride solutions. Before the dilution, the enzyme solution was dialyzed for several days in a collodion bag against distilled water, whereby most of the calcium salts must have disappeared. Using the experiment with the lowest calcium chloride concentration (0.0001 N, pH = 6.5), we get a value for pK of about 4.5.

Another series of experiments was made in the following way. A 1 % solution of an amylase preparation, containing some calcium salt, was precipitated with an excess of potassium oxalate at pH 6.5. The precipitate was centrifuged off and discarded and the supernatant precipitated with 1.1 volumes of acetone. The amylase thereby precipitated was centrifuged off and dissolved in water. Samples of this amylase solution were diluted 100 times with calcium chloride solutions of various strengths and an acetate buffer of pH = 5.0 was added (final ionic strength in the mixture $\mu = 0.003$). The samples were immersed in a thermostat at 30° C. The results of the activity measurements (6) are given in Table 1. They give a pK value of about 6.

Table 1

Inactivation of the amylase at various calcium salt concentrations.

$CaCl_2$ Normality	Activity after				pH
	0 h	1 h	3 h	20 h	
0	11.4	6.1	2.8	0.5	5.15
0.00001	12.5	11.5	9.8	4.5	5.20
0.0001	13.6	12.8	13.0	12.9	5.20

SUMMARY

For enzymes that can be considerably stabilized by metal ions, and whose inactivation is a first-order process the following expression is calculated for the dissociation constant K of the compound between the metal ion and the

active part of the enzyme molecule: $K = [\mathrm{M}]/(k_\mathrm{u}/k - 1)$, where k is the rate constant for the inactivation at the metal ion activity [M] and k_u the rate constant for the unstable enzyme. An accurate assay of this constant for the stabilization of malt α-amylase with calcium salts is difficult to carry out, for the value of pK is probably about 5 or 6.

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On the adiabatic compressibility of aqueous solutions

By BERTIL JACOBSON

With 13 figures in the text

Contents

Notation 177
Introduction 178
Chapter I. A theory for compressibility of solutions 179
  The ideal state 180
  Associated solutions 181
  Electrostriction 184
Chapter II. Experimental methods employed 186
  Method for determining sound velocity 186
  Density determinations 189
  Concentration determinations 189
  Chemical purifications and preparations 189
Chapter III. Experiments with associated solutions 190
Chapter IV. Experiments with solutions having electrostriction 197
Chapter V. General observations on solutions of macromolecules 201
Chapter VI. Proteins and desoxyribonucleic acid 204
Summary 209
References 210

Notation

B = $f_2/f_1\,(V_1/V_2)$, caracteristic constant for each solution.
b = van der Waal's constant.
c = molar concentration.
E = $V - \Phi$, electrostriction.
e = $cE/1000$, alteration in volume fraction of solvent with electrostriction.
f = form factor, gives the ratio between a molecular surface and the spherical surface which encloses a volume of size equal to the molecule.
G = ratio between the number of hydrogen bonds broken when cavities are formed in the solvent for the molecules of the solute and the total number hydrogen bonds possible in the solvent.
K = $(1 - v)\,(1 - \vartheta)\,G\,\xi$, correction term for the dependence of compressibility on the breaking of the association of the solvent through surface interaction.
N = Avogadro's number.

s = $b/4\,V$, space ratio.
V = (real) molar volume. V_1 and V_2 molar volumes of solvent and solute respectively.
v = $cV/1000$, volume fraction of solute.
Y_m = total internal molecular surface per mol.
Y_1, Y_2 = total internal molecular surface of solvent and solute respectively.
y' = surface of a spherical molecule.
y = surface of molecule of any random shape.
β = adiabatic compressibility of a solution.
β_1 = adiabatic compressibility of the solvent.
β_2 = adiabatic compressibility of the solute (for solids in hypothetical liquid state at the given temperature).
β_{mole} = adiabatic compressibility of a molecule.
$\triangle\beta$ = $\beta - \beta_1$, compressibility increment (decrement).
$\triangle\beta/c$ = molar compressibility increment (decrement).
$\triangle\beta/v$ = total compressibility increment (decrement).
Γ = $d\,(\triangle\beta/v)/dv$.
δ = difference in the total compressibility increment with and without electrostriction.
ζ = $d\beta/dv_e$, the compressibility alteration factor with electrostriction.
ϑ = factor which indicates the grade of association between solute and the water structure; fraction hydrogen bonds between solute and water per molecular surface of solute in relation to the mean value of hydrogen bonds in water for an equally large internal surface of water.
μ = constant expressing the mutual association tendency of the dissolved molecules in relation to the association tendency with solvent; v^{μ} is the part of the internal surface of the solute that does not come in contact with the solvent, in relation to the total internal surface of the solute.
ξ = compressibility alteration factor for breaking of the association of the solvent.
ϱ = density at 20° C in relation to water at 4° C.
Φ, Φ_0 = apparent molar volumes in solution and at infinite dilution respectively.

Introduction

The compressibility β' is a thermodynamic magnitude defined as the change in the volume v' per unit of volume for a given change in the pressure p.

$$\beta' = -\frac{1}{v'}\,\frac{\partial v'}{\partial p} \qquad (1)$$

The compression may be performed at constant temperature or constant entropy. In the former case the compressibility is designated isothermic and in the latter case isoentropic or adiabatic. The isothermic compressibility β_{is} may be related to the adiabatic β_{ad} by the ratio $\gamma = c_p/c_v$ between the specific heats at constant pressure and volume.

$$\beta_{\text{is}} = \gamma\,\beta_{\text{ad}} \qquad (2)$$

In the discussion which follows the adiabatic compressibility will exclusively be treated and we shall use $\beta_{\text{ad}} = \beta$.

Compressibility is measured in reciprocal units of pressure, e.g. at^{-1} and cm^2/dyne. To convert the figure for compressibility expressed in at^{-1} to cm^2/dyne the value in at^{-1} is divided by 1 013 300.

The adiabatic compressibility is best determined experimentally by measuring the sound velocity u and the density ϱ, after which it can be computed from the general hydrodynamic formula.

$$\beta = \frac{1}{u^2 \cdot \varrho} \tag{3}$$

The relation between compressibility and molecular structure for liquids has been studied in detail both theoretically and experimentally.[1] Special attention has not, however, usually been given to the compressibility direct but to the sound velocity, as the latter is easier to determine experimentally. A common feature of most theoretical computations is that the sound velocity and the compressibility are mainly determined from the space ratio $s = b/4\,V$. This space in turn is determined by the intermolecular properties and not by the intramolecular. As many problems concerning intermolecular biochemical processes are still unsolved, it would seem that a study of the compressibility of solutions may be of interest. Besides the general interest thus attaching to these problems, the present investigation has been undertaken to discover whether it is possible by means of compressibility measurements to obtain increased knowledge of the value of the elasticity modulus of the proteins. This constant is of fundamental importance to enable the position of the radio frequency spectra of the proteins to be computed.

Chapter I. A theory for compressibility of solutions

A number of research workers have studied sound velocity and compressibility in solutions and mixtures of liquids. Nevertheless, it has not been possible in general to draw any conclusions from the measurement results, which is due to the fact that the conditions with mixtures are more difficult to survey than with pure liquids, as the substance dissolved might be capable of causing change in the properties of the dissolving medium. (WILSON and RICHARDS (7), PARTHASARATHY (8), SMITH and EWING (9), WILLIS (10), GIACOMINI (11), BURTON (12).)

Such measurements have, however, furnished certain results. SCHAAFFS (13) states that the sound velocity of a very soluble substance may be obtained by extrapolation of the sound velocity in solutions of the substance in increasing concentrations, in spite of the curve for sound velocity versus concentration not being linear. This method of procedure, however, appears only to be possible when the sound velocity of the solute is close to that of the solvent. RICHARDS and PALITZSCH (14) 1919 studied the compressibility of aqueous solutions and state a minimum in compressibility for urethan solutions of rising concentration and explain it with a depolymerisation of the water. This problem has been discussed in modern form thoroughly by PARSHAD (15, 16, 17). In alcohol water solutions there occurs a mutual depolymerisation of both alcohol and water. The hydrogen bonds of water are broken and the water molecules then approach each other, resulting in reduced compressibility, which may be seen from a simple observation of the cohesive energy. This increases with the inverted value of the 6th power of the intermolecular distance, with the result that it becomes more and more difficult to displace a molecule from equilibrium the smaller the distance is and the compressibility will consequently be low. PARSHAD (18) has also indicated the possibility of deciding whether dipolar association or association with hydrogen bonds predominate in a solution. In analogy with the above reasoning the compressibility will in the former case be lower and in the latter case higher than theoretical anticipations.

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[1] The study of sound velocities in liquids and the adiabatic compressibility computed from them has provided considerably increased knowledge of the physical chemical properties of liquids. Thus a number of characteristic constants may be computed on the basis of such measurements. The relation between the specific heats c_p/c_v, the computation of van der Waal's b (SCHAAFFS (1)), molecular radii (SCHAAFFS (2)), space ratio $s = b/4\,V$ (SCHAAFFS (3, 4)), molecular weights in homologous series (WEISSLER and ref. (5)) and molar sound velocities (RAO (6)) are a few of the essential constants that may be determined in this way.

There does not appear to have been undertaken, however, any attempt at quantitative study for systematic treatment of the measurement results. The aim here is to try as a first approximation to find an expression for the compressibility of solutions, considering aqueous solutions in the first place, as these are of the greatest biochemical interest. It will be evident from the start that a number of approximations and hypothetical assumptions must be adopted, the accuracy of which can be tested and progressively corrected by thorough experimental investigation.

As the compressibility is a function of the intermolecular forces, we must deal with three different cases. First, the forces between the molecules of the solute and of the solvent may be a mean of the forces between equal molecules. This represents the ideal case and can only be expected to be correct in non-associated liquids and solutions. In the second case, when association is present, account must be taken of the degree of association in the solution. As a third possibility we shall deal with the case where the solute alters the intermolecular forces of the solvent by electrostriction. In further discussion there will be understood by solvent the low-molecular component in a binary system and by solute the high-molecular component, even if this is a liquid. It should also be observed that when the high-molecular component is a solid substance in pure state, there is understood here by its compressibility the compressibility of a hypothetical liquid at the temperature in question. The compressibility of the substance in crystalline state is of no interest in this connection.

A. The ideal state

In the ideal state when the intermolecular forces between solute and solvent is a mean of the forces between the different kinds of molecules the compressibility will vary linearly with the concentration, as per figure 1, curve B. If β_1 is the compressibility of the solvent and β_2 the compressibility of the solute, the resulting compressibility β may be derived from the equation.

$$\beta = v\,\beta_2 + (1 - v)\,\beta_1 \tag{4}$$

where v is the volume fraction of the solute. If c is the molar concentration and V the molar volume in ml then $v = cV/1\,000$.

For our experimental study it has been found advisable not to study the absolute value for β but the compressibility change $\triangle\beta$ per unit of concentration which arises through the addition of the substance.[1]

If $\triangle\beta = \beta - \beta_1$ is inserted in (1) we get

$$\frac{\Delta\beta}{v} = \beta_2 - \beta_1 \tag{5}$$

$\triangle\beta/v$ is the total compressibility increment or decrement for 100 vol. % solution, i.e. pure dissolved substance in hypothetical liquid state at the temperature in question.

B. Associated solutions

When the molecules of the solvent are bound to each other by association and the dissolved molecules have not a corresponding association tendency to the solvent the equations (4) and (5) are not applicable. Nor are the equations valid when association arises between the two substances without such existing in their respective pure states. To obtain a valid formula we shall add to eq. (4) a correction term K, which will then give the change in compressibility in a solution due to the prevailing association condition.

$$\beta = v\,\beta_2 + (1 - v)\,\beta_1 + K \tag{6}$$

There are two kinds of association, dipolar association and association with hydrogen bonds. According to PAULING (19) it is not possible, however, to draw a sharp line between the two terms owing to the ionic character of the hydrogen bonds. Here we shall only take into account association of hydrogen bonds as our knowledge of the association structure of water is entirely based on this method of observation. According to BERNAL and FOWLER (20) water may be regarded as a partially broken down ice structure and the latter has a tridymite-like structure with each oxygen atom surrounded tetrahedrally by four other oxygen atoms at a distance of 2.76 Å. The structure is held together by hydrogen bonds O—H $\cdots$ O.

To introduce a molecule of solute into the water structure a cavity must first be formed in this by taking away a given number of water molecules. To do this there must be broken a number of hydrogen bonds proportional to the size of the molecule surface y_2 of the solute and equal in number to the ratio between y_2 and the section of surface in the cavity which pertains to each hydrogen bond. It is, how-

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[1] For the study of the compressibility of solutions it is usual to employ as unit the apparent molar compressibility, defined as

$$\Phi\,(K_2) = (\beta v' - \beta_1 v'_1)/n \tag{i}$$

where β and β_1 and v' and v'_1 are the compressibilities and volumes respectively of the solution and the solvent, and n is the number of mols of solute. This does not seem so good for the reason that the apparent molar compressibility is dependent on the apparent molar volume $\Phi\,(V_2)$ and its variation with the concentration. This is best conveyed if $\Phi\,(K_2)$ is written as

$$\Phi\,(K_2) = \Phi(V_2)\,\beta_1 + 1\,000\,\triangle\beta/c \tag{ii}$$

where $\triangle\beta = \beta - \beta_1$ and c is the molar concentration. It would be more advisable to express the compressibility of a solution as the total compressibility increment $\triangle\beta/v$ or the corresponding molar increment $\triangle\beta/c$.

ever, the breaking of hydrogen bonds that causes change in compressibility and we should find out the number broken in relation to all those present in the water. This relation G times the compressibility change factor ξ is proportional to the wanted correction term K. ξ is the change in compressibility which follows on the breaking of hydrogen bond association from fully formed association at the temperature considered. It is assumed that ξ is constant for highly developed water structure, when water is the continuous phase. For higher concentration of solute and when the water structure is not continuous, ξ cannot be constant.

Here we shall only consider the breaking of the water structure and shall neglect the breaking of the association of the solute. K will then be proportional to the amount of water in the solution $(1 - v)$. Neglect of the solute's association does not appear to involve any great error when water is the solvent, as water contains more hydrogen bonds per unit of volume than other substances. Moreover it is probable that hydrogen bonds are formed between the water and the solute if this in itself is associated, and so this "breaking" does not have an equivalent change of compressibility. Instead there must be taken into account a mutual association between solute and solvent.

In actual fact, for an organic substance to be soluble in water requires that it possesses electro-negative atoms on its surface, which can be expected to form hydrogen bonds with water. Let ϑ designate a hydrogen bonding factor which gives the fraction bonds between water and solute per molecular surface of solute in relation to the mean value for water on an equally large inside surface of water at the temperature considered. When $\vartheta = 1$, the number of hydrogen bonds is equal to the mean value of water, i.e., no bonds are broken when the molecule is introduced in the water and no change in compressibility will occur. In the theoretical case where no association prevails between water and solute $\vartheta = 0$. The term $(1 - \vartheta)$ gives the degree of breaking of association and we can then write

$$K = (1 - v)(1 - \vartheta)\, G\, \xi. \tag{7}$$

To arrive at G we consider the inside surfaces in a solution. The inside surface in a liquid can easily be obtained as function of the molar volume V and the form of the molecules.

A sphere has the least possible surface in relation to the volume it encloses. A molecule of another form has a surface that can be related to the spherical by means of a form factor $f \geq 1$, which gives the relation between the molecule's surface and the imagined spherical surface which encloses the same volume as the molecule. The surface y' of a spherical molecule is thus obtained. The volume of a molecule is V/N, where N is Avogadro's number. The molecule radius is

$$r = \left(\frac{3\, V}{4\, \pi\, N}\right)^{\frac{1}{3}} \tag{8}$$

and the surface

$$y' = \left(36\, \pi \frac{V^2}{N^2}\right)^{\frac{1}{3}} \tag{9}$$

As the surface of any molecule is $f y'$ the inside surface Y_m in one mol of the substance will be

$$Y_m = N f y' = f (36\, \pi\, N\, V^2)^{\frac{1}{3}} \tag{10}$$

As the number of mols of solute per ml solution is v/V_2 the total surface of the solute is $Y_2 = Y_{m_2} v/V_2$ and the surface of the water $Y_{m_1}(1-v)/V_1$.

With higher concentrations a certain part of the surface of the solute will be ineffective in the breaking of hydrogen bonds, as the surfaces of the molecules touch each other and do not come into contact with the solvent. This reduction of the total surface Y_2 is difficult to estimate depending inter alia on the mutual association tendency and form of the molecules. The surface reduction should be a function of the volume fraction v, and we write this function as v^μ, where μ is a constant for each substance in a given solution, with a numerical positive value. When $\mu > 1$ the mutual association tendency of the dissolved molecules is small and when $\mu < 1$ it is great.

We now get

$$G = \frac{Y_2(1-v^\mu)}{Y_1} = v\frac{(1-v^\mu)}{(1-v)}B \tag{11}$$

where $B = f_2/f_1 (V_1/V_2)^{\frac{1}{3}}$ is a constant for a given type of solution.

With the aid of eq. (7) and (11) we obtain from (6) the wanted expression for compressibility

$$\beta = v\beta_2 + (1-v)\beta_1 + v(1-v^\mu)(1-\vartheta)B\xi \tag{12}$$

β will vary with the concentration as curve A in figure 1. The absolute minimum or maximum in compressibility will occur at a concentration v_m which is obtained from $d\beta/dv = 0$

$$v_m = \left[\frac{\beta_2 - \beta_1}{(1+\mu)(1-\vartheta)B\xi} + \frac{1}{1+\mu}\right]^{\frac{1}{\mu}} \tag{13}$$

β is minimum if $(1-\vartheta)\xi$ is negative, which always is the case for aqueous solutions.

We also get

$$\frac{\Delta\beta}{v} = \beta_2 - \beta_1 + (1-v^\mu)(1-\vartheta)B\xi. \tag{14}$$

Where ξ is negative, i.e., the compressibility decreases on breaking of the association the term $(1-v^\mu)(1-\vartheta)B\xi$ will be negative for $\vartheta < 1$. The absolute magnitude of the term will be proportional to $(1-v^\mu)$ so that it decreases with rise in concentration and $\Delta\beta/v$ approaches $\beta_2 - \beta_1$ when $v = 1$ according to figure 2.

To obtain an expression for μ there is formed

$$\Gamma = \frac{d(\Delta\beta/v)}{dv} = -\mu v^{\mu-1}(1-\vartheta)B\xi \tag{15}$$

If two volume fractions v_a and v_b have the slopes Γ_a and Γ_b there applies

$$\mu = \frac{\log\frac{\Gamma_a}{\Gamma_b}}{\log\frac{v_a}{v_b}} + 1 \tag{16}$$

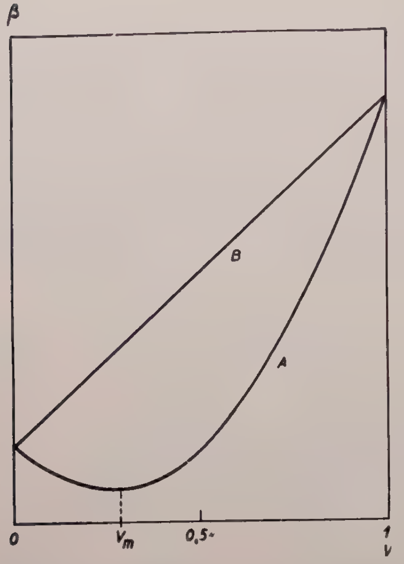

Fig. 1. Compressibility as a function of the fraction by volume of solute. Curve B represents the ideal case according to formula (4) and curve A the case when association exists according to (12).

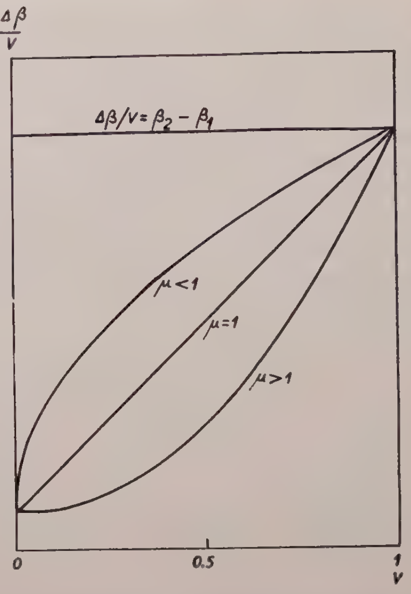

Fig. 2. Total compressibility increment as a function of the fraction by volume of solute at different values of μ according to formula (14).

When $v \to 0$ then Γ assumes different values depending on μ. If $\mu > 1$ then $\Gamma \to 0$, when $\mu = 1$ then $\Gamma = (1 - \vartheta) B \xi$ and when $\mu < 1$ then $\Gamma \to \infty$ as is shown in figure 2.

C. Electrostriction

When the solute is charged electrically, for example, through a permanent dipole moment, the solvent's molecules will be more densely packed owing to the action of the electric field. The decrease in volume $E = V - \Phi$ is the electrostriction in the solvent if V is the real molar volume of the substance and Φ is the apparent molar volume in the solution. When $\Phi = \Phi_0 + ac$, where Φ_0 is the apparent molar volume for infinite dilution, the electrostriction will vary to some extent with the concentration c, but as a is a characteristic constant for each substance in a given solvent with a numerically small value, E can approximately be regarded as constant for varying concentration.

Owing to the denser packing of the solvent's molecules with electrostriction the compressibility will diminish. We shall here assume this diminution to be proportional to the decrease in volume

$$\frac{d\beta_e}{dv_e} = \zeta \tag{17}$$

where β_e and v_e are the compressibility and volume fraction of the water with electrostriction and ζ is thus a constant. This assumption corresponds with the above-stated approximative linear relation between electrostriction and concentration. Each dipole produces only a compressing of the water in the vicinity of the molecule and each addition of solute produces an electrostriction proportional to the increment of concentration. The change in compressibility will then be also proportional to the concentration. This is valid only for solutions of polar substances with low concentration. With electrolytes, where the electric field is formed between ions whose distance apart changes with the concentration, the electrostriction cannot be proportional to the concentration. With polar solutions of high concentration when dipole-dipole interaction occurs equation (17) cannot be valid. We confine ourselves to the study of low concentrations.

To obtain β_e as a function of the concentration we utilise $dv_e/dc = -e/c$ which is valid if $e = cE/1\,000$ is the electrostriction factor and when $v_e = (1 - e)$. We get

$$\frac{d\beta_e}{dc} = -\frac{\zeta E}{1000} \tag{18}$$

and

$$\beta_e = \beta_1 - \zeta e \tag{19}$$

when the integration constant will be β_1.

As the new volume fraction of the water is $(1 - v - e)$ there is obtained

$$\beta = v\beta_2 + (1 - v - e)\beta_e \tag{20}$$

or if (19) is utilised and e^2 is neglected in comparison with e

$$\beta = v\beta_2 + (1 - v - e)\beta_1 - e\zeta(1 - v) \tag{21}$$

and

$$\frac{\Delta\beta}{v} = \beta_2 - \beta_1 - \frac{e}{v}(\zeta(1 - v) + \beta_1) \tag{22}$$

When $e/v = E/V$ and is thus constant $\triangle\beta/v$ will vary linearly with the concentration with the slope

$$\Gamma = \frac{E}{V}\zeta \tag{23}$$

For the experimental study it has been found advisable to keep electrostriction and breaking of the hydrogen bond association through surface interaction entirely apart and assume that they do not have a mutual action on each other as regards change of compressibility in a solution. We leave on one side the deeper nature of the electrostriction for the time being. The complete expression for the compressibility will be

$$\beta = v\beta_2 + (1 - v - e)\beta_1 + v(1 - v^u)(1 - \vartheta)B\xi - e\zeta(1 - v) \tag{24}$$

and

$$\frac{\Delta\beta}{v} = \beta_2 - \beta_1 + (1 - v^u)(1 - \vartheta)B\xi - \frac{E}{V}(\beta_1 + \zeta(1 - v)) \tag{25}$$

The difference δ in value for $\triangle\beta/v$ for a substance with and without electrostriction will be obtained if (25) is subtracted from (14)

$$\delta = \frac{E}{V}(\beta_1 + (1 - v)\zeta) \tag{26}$$

Chapter II. Experimental methods employed

The adiabatic compressibility in solutions may be determined with great accuracy by measurement of the sound velocity and the density, the compressibility then being obtained from formula (3). The sound velocity u is best measured by measuring the wave length λ in the solution; when the frequency ν is known, there is obtained $u = \nu\lambda$.

Method for determining sound velocity

The sound velocity in the solutions investigated was obtained by using an ultrasonic interferometer according to PIERCE (21), working with a frequency of 1 Mc/sec (figure 4). The wave length of standing waves in the solution between an ultra-sonic quartz plate and a reflecting surface is measured by micrometer setting of the reflecting surface. To indicate the accoustic resonance in the solution, there is utilised the alteration in the ultra-sonic oscillator frequency, which arises when the position of the reflecting surface is altered from resonance position. Block diagram of the electric units is given in figure 3. The frequency alteration is studied by mixing the ultra-sonic oscillator frequency with a crystal controlled frequency of app. 1 Mc/sec. The low frequency beat thus arising is made to pass a low-pass filter, after which the voltage is measured with an AC voltmeter (figure 5). The maximum deflection of the voltmeter is equivalent to acoustic resonance in the interferometer. Figure 6 shows the characteristics of the filter and figure 7 a typical resonance peak in the interferometer studied with the aid of the voltmeter deflection.

To determine the exact frequency ν of the ultra-sound, the frequency of the crystal-controlled oscillator was calibrated exactly with the aid of broadcasting stations. To obtain the wanted ultra-sound frequency it is therefore only necessary to measure the low frequency beat at each measurement. This is done with a frequency meter consisting of a low-frequency oscillator and a cathode ray tube, the periodicity of the beat being determined by means of Lissajou's figure. To facilitate the measurement the low frequency beat was made audible by connecting a loud-speaker.

The advantages of the apparatus employed over those earlier described are as follows:

1) The requisite amount of liquid for the interferometer is only 4.5 ml as against ten times as much with earlier designs for the same frequency. It is not possible to bring down the requisite volume of solution still further at the same frequency

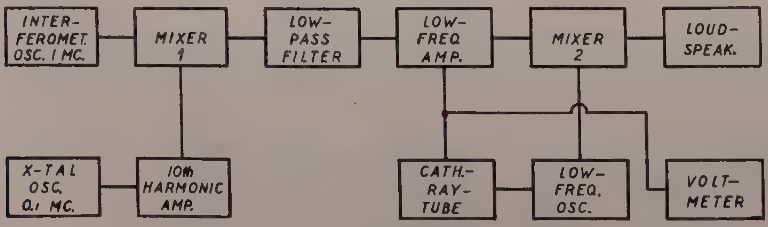

Fig. 3. Block diagram showing the electric units.

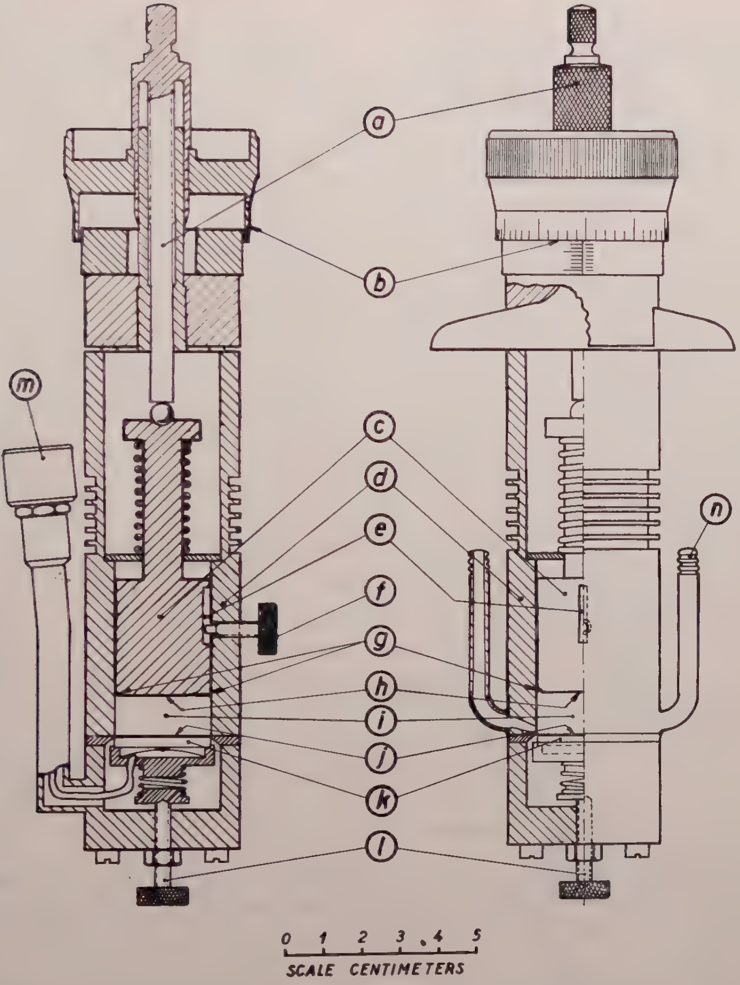

Fig. 4. Ultra-sonic interferometer. *a* micrometer, type C. E. Johansson 126, *b* measuring drum with divisions for readings of $1 \cdot 10^{-3}$ mm. *c* piston moving in cylinder *d*. *f* screw running in the groove *e* to prevent twisting of the piston. *g* bevelling in piston to prevent occurrence of spurious oscillations and to collect small air bubbles. *h* reflector surface in the standing wave chamber *i*. *j* steel diaphragm 0.05 mm thick. *k* piezoelectric ultra-sonic crystal for 1 Mc. *l* screw for regulation of the crystal's pressure on the diaphragm. Acoustic coupling between crystal and diaphragm is obtained by means of a small quantity thin oil. *m* coaxial contact for high frequency output to crystal. *n* filling pipe. All parts coming into contact with the solution are made of stainless steel.

and accuracy. 2) The method employed for indicating the change of frequency approaching accoustic resonance makes possible rapid measurement and maximum accuracy, besides the employment of low ultra-sonic intensity. 3) The low ultra-sonic intensity is obtained by the extremely low anode voltage in the oscillator valve 6C5 of approximately 15 Volts. In this way the power developed in the solu-

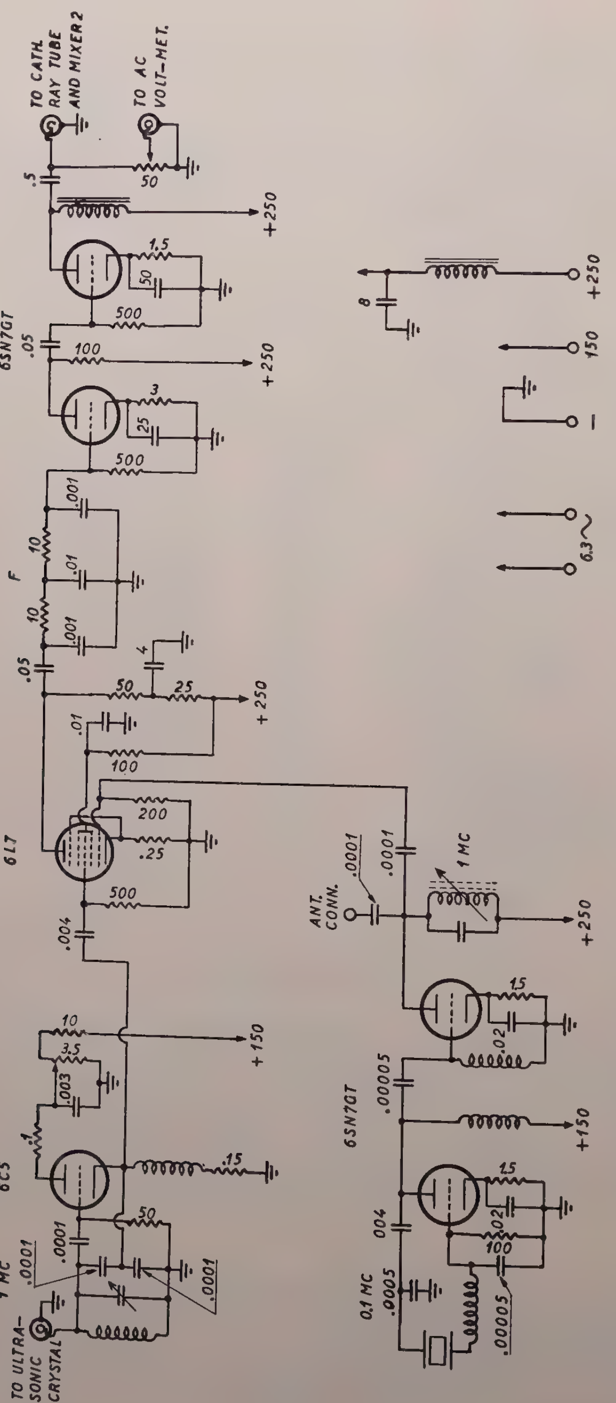
TO ULTRA-SONIC CRYSTAL
1 MC
.0001
.0001
.0001
6C5
1
.003
3.5
10
50
.15
+150
.004
500
.25
200
6L7
100
.01
50
25
4
+250
.05
10
.001
.01
10
.001
F
500
25
3
100
+250
.05
6SN7GT
500
50
1.5
.5
50
+250
TO CATH. RAY TUBE AND MIXER 2
TO AC VOLT-MET.
ANT. CONN.
.0001
.0001
1 MC
+250
6SN7GT
.00005
1.5
.02
+150
1.5
.02
100
.004
0.1 MC
.0005
.00005
8
+250
150
–
6.3 ~

Fig. 5. Circuit diagram of the electric units except the frequency meter. Ultra-sonic oscillator 6C5, crystal controlled oscillator and harmonic amplifier 6SN7GT, first mixer 6L7, low-pass filter F and low-frequency amplifier 6SN7GT.

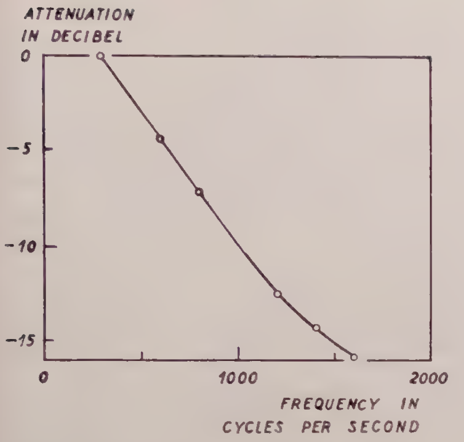

Fig. 6. Attenuation caracteristic of low-pass filter F.

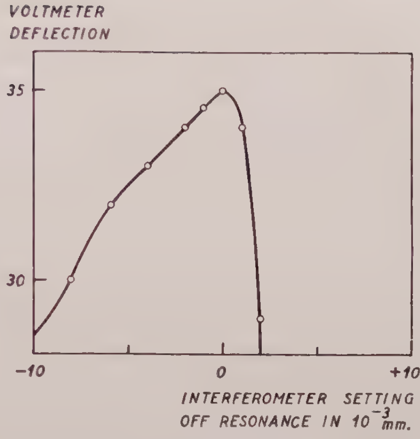

Fig. 7. Typical resonance peak in the ultra sonic interferometer.

tion is brought down to less than $5 \cdot 10^{-4}$ watt, which corresponds to a maximum temperature rise with the smallest possible volume of solution in the interferometer of 10^{-2} °C per minute. This is important, as the measurement accuracy increases with increased stability of temperature and as chemical changes of the solution due to heating, cavitation and formation of H_2O_2 are avoided.

The absolute accuracy of the method is determined by the accuracy of 0.2 % of the micrometer screw. The relative accuracy is 0.05 % and is determined essentially by the temperature stabilisation of the interferometer.

Density determinations

The values for density in solutions which are required for computing compressibility were obtained by means of pycnometers of 25 and 8 ml respectively. The accuracy was always greater than the accuracy of the square of the sound velocity 0.1 %.

Concentration determinations

The initial concentrations for the highest concentrations of dissolved substances were obtained by weighing, except for the proteins when nitrogen analysis according to Kjeldahl was employed. With the successive dilutions the concentration was determined by weighing. The relative error in the different concentrations of a test series is in general negligible and is due to evaporation of the most volatile phase in the binary system. Only for the very volatile liquids such as pentane is the concentration determination uncertain. Nevertheless the errors cannot influence the final results obtained in the experiments.

Chemical purification and preparation

The liquids used in the experiments described in chapter III were of the highest commercial degree of purity. No further purification was considered necessary.

The only impurity that might essentially alter the compressibility data is water. It is seen from the density values, however, that in all cases this was of an order of magnitude which was without interest in this connection.

The amino acids used in the experiments described in chapter IV were re-crystallised once or twice. The initial material was of the highest commercial degree of purity. The glycolamide was synthesized according to HEINTZ (22) and the lactamide according to BRÜNING (23) and was recrystallised, melting points 118° C and 74.5° C respectively. The density values for solutions of these substances agree very well with the values obtained by other authors, thus indicating satisfactory purity.

The proteins used in the experiments described in chapter VI were prepared according to the following authors and the nitrogen values employed for concentration determinations are: Horse serum albumin crystallised according to ADAIR and ROBINSON (24), 15.6 % N; Oxyhaemoglobin from horses crystallised according to HEIDELBERGER and LANDSTEINER (25), 17.38 % N; and Haemocyanin from Helix Pomatia according to BROHULT (26), 15.15 % N; The protein solutions were dialyzed against distilled water.

The desoxyribonucleic acid was prepared according to HAMMARSTEN 1924 (27) and the analysis gave the following values: phosphorus 7.00 %, nitrogen 11.99 % and moisture 22.00 %. The crystalline desoxyribonuclease was supplied by the Worthington Biochemical Laboratory, Freehold, New Jersey.

Chapter III. Experiments with associated solutions

With a view to testing the correctness of the formulae stated in the two first parts of chapter I a number of measurements have been carried out on the compressibility in mixtures of liquids. Only liquids that are mixable with each other in all proportions have been selected. In this way it is possible to follow the dependence of the compressibility on the concentration to its full extent.

1. The ideal state

Table 1

Experimental and calculated values for different fractions by volume of heptane in pentane at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0	1.0394	0.6278	147.44		
0.223	1.0584	0.6416	139.13	139.08	− 0.00
0.409	1.0823	0.6519	130.96	132.11	+ 0.88
0.607	1.1071	0.6622	123.21	124.67	+ 1.18
0.806	1.1301	0.6737	116.22	117.21	+ 0.85
1.000	1.1524	0.6849	109.94	Mean deviation = 0.73 %	

To throw light on the extent to which the compressibility in mixtures of non-associated liquids will be a linear function of the concentration a trial was made with pentane and heptane. The result will be seen in table 1. The computed compressibility was obtained from formula (4). The values taken for the compressibility of pentane (β_1) and heptane (β_2) were the values measured on pure liquids ($v = 0$ and $v = 1$). The agreement between the computed and the measured values may be regarded as satisfactory.

Table 2

Experimental and calculated values for different fractions by volume of methanol in water at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0	1.4865	0.9982	45.34		
0.098	1.5233	0.9847	43.77	43.88	+ 0.26
0.194	1.5566	0.9718	42.47	42.76	+ 0.69
0.315	1.5769	0.9554	42.09	42.34	+ 0.59
0.390	1.5691	0.9438	43.04	42.91	− 0.30
0.488	1.5393	0.9271	45.52	45.04	− 1.06
0.591	1.4865	0.9064	49.93	49.43	− 1.00
0.692	1.4175	0.8838	56.31	56.33	+ 0.02
0.790	1.3364	0.8588	65.20	65.47	+ 0.42
0.897	1.2374	0.8277	78.91	80.31	+ 1.78
1.000	1.1293	0.7949	98.64	Mean deviation = 0.68 %	

Table 3

Experimental and calculated values for different fractions by volume of ethanol in water at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0	1.4861	0.9982	45.35		
0.099	1.5491	0.9839	42.35	42.02	− 0.78
0.193	1.6046	0.9734	39.90	40.19	+ 0.72
0.313	1.6298	0.9571	39.33	39.97	+ 1.61
0.391	1.6025	0.9448	41.22	41.14	− 0.19
0.487	1.5470	0.9258	45.13	44.20	+ 2.08
0.589	1.4788	0.9037	50.60	49.44	− 2.29
0.686	1.4175	0.8847	56.25	56.44	+ 0.33
0.791	1.3399	0.8566	65.03	66.15	+ 1.73
0.896	1.2703	0.8283	74.82	78.20	+ 4.52
1.000	1.1682	0.7919	92.53	Mean deviation = 1.58 %	

Table 4

Experimental and calculated values for different fractions by volume of *n*-propanol in water at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0.0	1.4860	0.9982	45.37		
0.096	1.5580	0.9863	41.77	42.01	+0.58
0.193	1.5976	0.9748	40.19	42.19	+4.98
0.293	1.5538	0.9593	43.18	43.57	+0.91
0.390	1.5036	0.9411	47.00	46.07	−1.98
0.490	1.4547	0.9214	51.29	49.82	−2.86
0.588	1.4151	0.9014	55.40	54.52	−1.59
0.695	1.3702	0.8791	60.59	60.59	+0.00
0.792	1.3322	0.8580	65.67	67.01	+2.03
0.886	1.2918	0.8335	71.90	73.72	+2.54
1.000	1.2250	0.8045	82.83	Mean deviation = 1.94 %	

Table 5

Experimental and calculated values for different fractions by volume of iso-propanol in water at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0.0	1.4858	0.9982	45.38		
0.098	1.5621	0.9850	41.61	41.47	−0.33
0.195	1.6222	0.9735	39.04	40.20	+2.99
0.319	1.6000	0.9556	40.88	41.45	+1.39
0.392	1.5510	0.9417	44.14	43.52	−1.41
0.493	1.4874	0.9208	49.09	47.59	−3.06
0.593	1.4220	0.8980	55.07	54.00	−1.94
0.692	1.3600	0.8745	61.83	61.35	−0.77
0.795	1.3021	0.8490	69.47	70.53	+1.52
0.901	1.2386	0.8188	79.61	81.59	+2.49
1.000	1.1676	0.7871	93.19	Mean deviation = 1.77 %	

2. Associated solutions

For experiments with associated solutions there were taken mixtures of water and methanol, ethanol, n-propanol, iso-propanol and acetone. To interpret the measurement results (tables 2–6) and enable theoretical computation of the compressibility to be carried out from the formula (12), the constants, B, ϑ, μ and ξ must be discussed in these special cases.

Table 6

Experimental and calculated values for different fractions by volume of acetone in water at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0.0	1.4857	0.9982	45.39		
0.101	1.5412	0.9868	42.66	42.74	+0.00
0.194	1.5752	0.9758	41.30	41.29	−0.00
0.297	1.5905	0.9626	41.07	41.02	−0.00
0.396	1.5764	0.9476	42.47	42.25	−0.50
0.494	1.5407	0.9309	45.25	45.07	−0.41
0.595	1.4880	0.9105	49.60	49.78	+0.34
0.698	1.4209	0.8879	55.78	56.63	+1.52
0.789	1.3568	0.8640	62.87	64.36	+2.36
0.898	1.2785	0.8316	73.57	75.96	+3.16
1.000	1.1898	0.7929	89.09	Mean deviation = 0.92 %	

Table 7

Theoretically and experimentally determined constants for various liquids in mixtures with water at 20° C.

Liquid	V_2 ml	f_2	B	ϑ	μ	ξ 10^{-12} cm^2/dyne
Methanol	40.41	1.00	0.765	0.29	2.1	−127
Ethanol.........	58.37	1.01	0.683	0.22	1.2	−162
n-propanol	74.70	1.04	0.648	0.19	0.45	−196
iso-propanol	76.51	1.00	0.618	0.19	0.70	−218
Acetone.........	73.33	1.00	0.627	0.20	1.3	−146
					ξ mean = −170	

Computation of constant B. According to the formula (11) $B = f_2/f_1 (V_1/V_2)^{\frac{1}{3}}$. As the solvent is water, V_1 assumes the value of 18.05 at 20° C. For the other liquids V_2 is given in table 7.

The form of the water molecule has been assumed as being spherical, so that $f_1 = 1$. The form factors f_2 for the other substances have been estimated in the manner below and are given in table 7. The form of the methanol molecule is also regarded as spherical. For ethanol and n-propanol f_2 has been determined by assuming a cylindrical molecule form with methanol's radius and with the bounding surfaces at both ends semi-spherical. By analogous computations for iso-propanol and acetone it is found that the form factor cannot depart very much from 1. B has been computed from the values thus computed for form factor and molar volume.

Discussion of hydrogen bonding factor ϑ. ϑ designates the fraction of hydrogen bonds between the water and the solute per inside molecule surface of solute in relation to the mean value of water for an equally large inside surface of water.

TAFT and SISLER (28) calculated from melting heat and vaporising heat that on melting app. 11 % and on rise of temperature from 0 to 100° C app. 16 % of the total number of possible hydrogen bonds in the water structure are ruptured. Thus at 20° C there remains app. 86 % of the total number possible, which represents an average of 1.7 bonds per oxygen atom. These take up half the water molecule's surface of 46.79 Å². Each hydrogen bond, therefore, at 20° C takes up an average inside molecule surface of water of 13.6 Å².

The liquids now investigated have one oxygen atom exposed for the formation of hydrogen bonds with water. The number of hydrogen bonds with this oxygen atom cannot be decided definitely, but it cannot be larger than 2 and probably not smaller than 1. We shall assume an equally great hydrogen bonds association with the solute's oxygen atom as with the water's oxygen atoms and take 1.7 as a reasonable number. From this assumption ϑ may be computed.

Experimental determination of μ. On the basis of equation (12) it was assumed that the reduction of effective internal surface of the solute was proportional to v^{μ}. μ does not lend itself to theoretical computation but it can easily be found from the experimental data in tables 2–6. For this reason $\triangle\beta/v$ has been computed and plotted as a function of v. The smoothed curve should be a generalised parabola, and this was approximately the case in the five experiments. As an example, figure (9) is given for methanol. From the slope Γ for two points, μ can be computed according to equation (16).

As stated in chapter I, $\mu > 1$ will mean that the molecules of the solute have great affinity to the water molecules and when $\mu < 1$ the affinity is small. In accordance with this μ should decrease for increasing length in the paraffin chain in the homologous alcohols. The iso-propanol should probably have a μ-value between those of ethanol and n-propanol. As may be seen from table 7, μ decreases in the order that may be assumed from the constitution formulae of the substances.

It is also of interest to compare the relative magnitudes of μ with the mixing heats obtained for the mixture of the different alcohols with water. According to LANDOLT BÖRNSTEIN (29), the heat is greatest for methanol, rather less for ethanol and least for n-propanol. The n-propanol has in volume fractions over 0.7 negative mixing heat. The high positive mixing heats are an expression of strong hydrogen bonds association with water. From the relative magnitudes of the mixing heats for the three alcohols, therefore, there is also obtained that μ should vary, as the experiments show.

It is worthy of note also that a relation should exist between μ and ϑ, with μ the dependent magnitude. To find out about this would seem, however, to require increased experimental experience.

Experimental determination of ξ. The factor of compressibility change ξ on breaking of the hydrogen bonds of the water structure can now be determined, as B, ϑ and μ are known. There has been taken the value for $v(1-v^{\mu})(1-\vartheta)B\xi$ which gave the best agreement between the compressibilities with varying concentrations found and computed from equation (12). In this way ξ obtains a given value for each substance and this is given in table 7. Taking into consideration the uncertainty lying in the determination of ϑ, μ and even to a certain extent of B, the variation present in ξ cannot be regarded as contradicting the correctness of equation (12). The mean value $\xi = -1.7 \cdot 10^{-10}$ cm²/dyne is a probable value at 20° C.

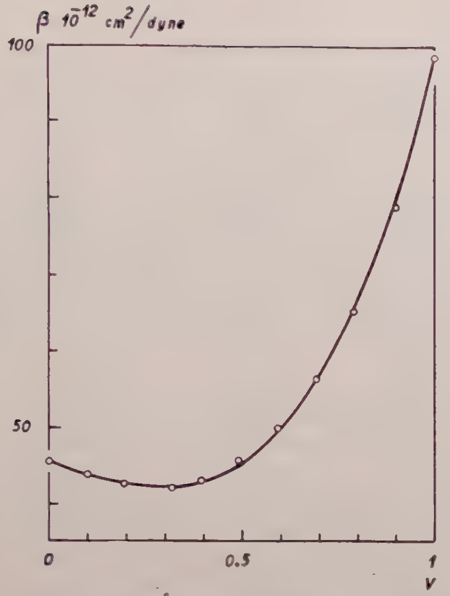

Fig. 8. Theoretically computed (unbroken line) and experimentally found (points) compressibility for different fractions by volume of methanol in water.

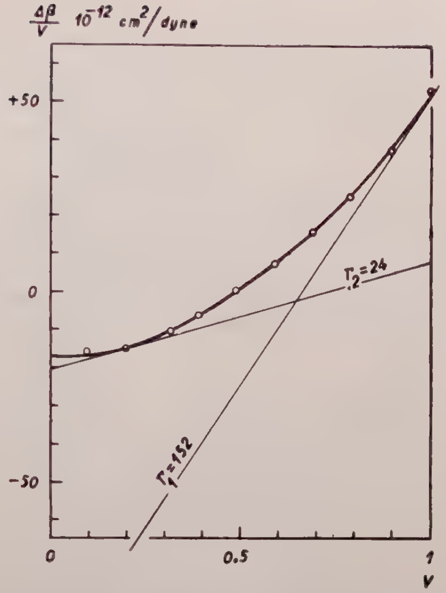

Fig. 9. Compressibility increment as a function of fraction by volume of methanol in water. From two slopes for different concentrations it is possible to compute the constant μ from equation (16).

Comparative estimation of the order of magnitude of ξ from the temperature coefficient of the water. Water is the only liquid which has a negative temperature coefficient for compressibility. Between 0 and 70° C the compressibility decreases for rising temperature. From values stated by RANDALL (30) it can be computed that water decreases its compressibility per degree C by app. 0.10 cm²/dyne. It must, however, be considered that the total decrease of compressibility is apparently of larger amount as liquids in general increase their compressibility through thermic expansion. This increase is relatively constant for non associated liquids. RAO (31) studied the variation in sound velocity and density at different temperatures for a number of liquids. From these values it can be reckoned that the percentages of increase in compressibility per degree of temperature rise at 20° C have the following values for the non associated liquids: carbon tetrachloride, +0.86, heptan, +0.91, and octan, +0.89 %. According to RAO (32), the compressibility increases for most liquids by 60 % for rise of temperature from 0 to 60°, which agrees well with the above. If it is assumed that water in a hypothetical non associated state would have a corresponding positive temperature coefficient of 0.9 %, we get that the compressibility of water at 20° C would increase by app. 0.40 cm²/dyne. If we add this to the decrease observed experimentally there is obtained a final decrease of app. 0.5 cm²/dyne per degree of rise in temperature.

From the values given by TAFT and SISLER (28) for percentage of rupture of hydrogen bonds in water at different temperatures it can be computed that app. 0.19 %

of the total number of possible bonds is broken per degree rise in temperature at 20° C.

According to the significance of ξ we get that

$$\xi = -0.5/0.0019 = -2.6 \cdot 10^{-10} \text{ cm}^2/\text{dyne}.$$

This value is, of course, very uncertain and serves only to illustrate that the same order of magnitude for ξ is obtained by this computation as by the experimentally determined $-1.7 \cdot 10^{-10}$. The agreement suggests the correctness of the assumption in deriving the formula (12) that breaking down of hydrogen bonds in water on dissolving a substance is a local process in the vicinity of the molecule surface of the solute.

Comparison between compressibilities computed and found. The values given in tables 2–6 for the computed compressibility were obtained from the formula (12), the same value for $(1-\vartheta) B\xi$ being taken for the various experiments as were utilised in computing ξ in table 7. The computed values are therefore only of value in investigating the relative agreement for different concentrations. The agreement is best for acetone and methanol and falls off for increasing length in carbon chain in the alcohols, but in all the cases it must be regarded as very good. As an example the measured and computed values for the experiment on methanol are given in figure (8).

In all cases the agreement is less good for the highest volume fraction (v app. 0.9). There is no reason to be surprised at this, as no continuous water structure can be supposed to be built up here.

Further discussion of ϑ. In the experiments described above ϑ had a small value, but this need not always be the case. If $\vartheta = 1$ the equation (12) is reduced to (4). $\vartheta = 1$ implies that equally great association prevails between the unlike molecules as between the like in a binary mixture. The possibility of finding such a system has been investigated. The mixing heat for such a mixture should, according to what was stated in the discussion of μ, be zero. It is then probable that no essential changes in the degree of association will arise on mixing. According to LANDOLT BÖRNSTEIN (29) the mixing heat for methanol and ethanol has a very small negative value, the absolute amount of which is less than 1 % of the amount for methanol-water. Methanol-ethanol should, therefore, be a suitable system for testing the above reasoning concerning ϑ, and compressibility has therefore been measured for such mixtures. The results will be seen in table 8. The computed values were obtained from formula (4) in analogy with the values for petane-heptane. The agreement is very good.

ϑ may also be greater than 1. In this case the compressibility curve according to equation (12) should be convex towards higher compressibility values. As was shown by PARSCHAD (18) the compressibility does have such a course with mixtures of acetone and chloroform. The author suggests the reason for this is an acetone-chloroform association with hydrogen bonds, which would not be present with pure liquids.

The significance given to ϑ in the discussion of the association of aqueous solutions would appear therefore as possible also of application to other liquids.

Table 8

Experimental and calculated values for different fractions by volume of ethanol in methanol at 20° C.

Fraction by volume	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne		Deviation %
			Observed	Calculated	
0.0	1.1301	0.7937	98.65		
0.247	1.1397	0.7940	96.96	97.18	+0.23
0.426	1.1458	0.7934	96.01	96.13	+0.13
0.499	1.1489	0.7933	95.50	95.69	+0.20
0.571	1.1514	0.7935	95.06	95.26	+0.21
0.761	1.1589	0.7929	93.91	94.14	+0.24
1.000	1.1673	0.7916	92.72	Mean deviation = 0.20 %	

General discussion. The good relative agreement between computed and measured compressibility for different concentrations in the experiments performed makes it probable that formula (12) and the assumptions made in derivation are valid. The formula contains only the concentration as variable. The other magnitudes B, ϑ, μ and ξ are constants with clear physical significance. B may be computed from known constants. ϑ could only so far be estimated theoretically. The value for ϑ, however, in the five experiments with water mixtures, is small and is contained in formula (12) only as $(1-\vartheta)$, so that the total error will not be large. μ has only been determined from compressibility measurements. The relative values obtained for μ agree well with the significance given to the constant. The values obtained for ξ as result of the different experiments do not conflict with ξ being a constant. It should, however, be observed that ξ refers to highly formed structure in water. When this structure is much broken down ξ is not constant. This is shown, inter alia, by the value for ξ being app. 4 times the total compressibility of water. ξ will also be very much dependent on the temperature.

As a working hypothesis we shall regard equation (12) as being valid and assume the value of $\xi = -1.7 \cdot 10^{-10}$ cm^2/dyne.

Chapter IV. Experiments with solutions having electrostriction

In the discussion of electrostriction in solutions in chapter I the assumption was made that this does not affect the association between the solute and the solvent. This condition is necessary in order to be able to relate the change of compressibility to a given electrostriction arising. The electrostriction has in fact been obtained experimentally as the difference between the apparent molar volume in solution of charged and uncharged isomers and in this determination no account is taken of different association conditions (Cohn and Edsall (33)).

The constant ζ expressing the compressibility alteration with electrostriction is the only unknown magnitude in the formulae (19)–(26) of chap. I, which were given to relate the compressibility to the electrostriction in a solution. In order to

Table 9

Experimental values for different concentrations of glycolamide in water at 20° C.

Concentration mol per liter	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne
0.0		0.9982	45.34
0.193	1.4916	1.0019	44.86
0.388	1.4975	1.0058	44.34
0.579	1.5038	1.0095	43.80
0.847	1.5102	1.0147	43.21
1.173	1.5195	1.0211	42.42
1.401	1.5253	1.0254	41.92
1.623	1.5326	1.0297	41.35

Table 10

Experimental values for different concentrations of glycine in water at 20° C.

Concentration mol per liter	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne
0.0		0.9982	45.40
0.193	1.4958	1.0043	44.50
0.323	1.5037	1.0084	43.86
0.383	1.5065	1.0103	43.61
0.505	1.5127	1.0141	43.10
0.619	1.5197	1.0175	42.56
1.177	1.5483	1.0346	40.32
2.000	1.5907	1.0587	37.33
2.561	1.6186	1.0743	35.53

Table 11

Experimental values for different concentrations of lactamide in water at 20° C.

Concentration mol per liter	Sound velocity 10^5 cm/sec.	Density$^{20}_{4}$	Compressibility 10^{-12} cm^2/dyne
0.0		0.9982	45.35
0.127	1.4914	1.0001	44.95
0.232	1.4960	1.0018	44.60
0.419	1.5028	1.0047	44.07
0.609	1.5105	1.0077	43.49
0.903	1.5227	1.0123	42.61
1.370	1.5406	1.0198	41.32
1.687	1.5522	1.0246	40.51

Table 12

Experimental values for different concentrations of α-alanine in water at 20° C.

Concentration mol per liter	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne
0.0		0.9982	45.36
0.105	1.4930	1.0014	44.80
0.197	1.4996	1.0041	44.29
0.354	1.5100	1.0085	43.49
0.573	1.5250	1.0146	42.38
0.841	1.5429	1.0221	41.10
1.223	1.5681	1.0321	39.40
1.579	1.5916	1.0420	37.89

Table 13

Experimental values for different concentrations of β-alanine in water at 20° C.

Concentration mol per liter	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne
0.0		0.9982	45.30
0.115	1.4947	1.0019	44.68
0.253	1.5039	1.0062	43.94
0.474	1.5186	1.0128	42.81
0.757	1.5370	1.0213	41.45
1.119	1.5607	1.0318	39.79
1.571	1.5890	1.0446	37.91
2.140	1.6270	1.0603	35.63

determine its value there should be measured the compressibility in solutions of substances with the same molecular weight and the same composition percentage but with different permanent dipole moments. Then the space ratio $s = b/4\ V$ will be as near as possible constant as regards the solutes. The dipole-dipole interaction can be entirely ignored if the measurements are made in diluted solutions. The only difference in the space ratio depends on the denser packing of the molecules of water owing to electrostriction. The difference in compressibility will then be entirely dependent on the electrostriction.

Compressibility in solutions of isomers. For the experimental studies we have taken the isomers glycine and glycolamide and also α-alanine, β-alanine and lactamide. The results of measurements of compressibility in solutions of these substances for different concentrations are given in tables 9–13.

For the uncharged substances glycolamide and lactamide $\Delta\beta/v$ was, within experimental limits of error, a linear function of the concentration. The experimental errors, however, grow strongly as one approaches low concentrations owing to the very slight difference $\Delta\beta$ between the compressibility of the solution and that of water.

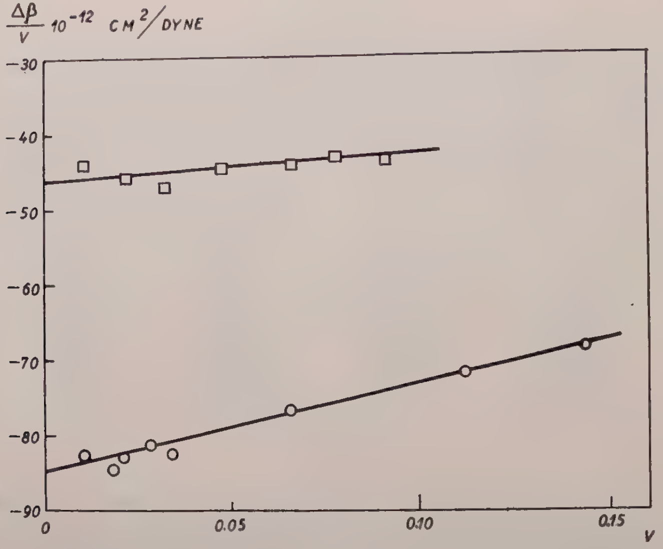

Fig. 10. Total compressibility decrement as a function of fraction by volume of glycolamide (□) and its isomer glycine (○).

If $\triangle\beta/v$ versus v is linear for the uncharged isomers the theory requires that there shall be linearity also in respect of the charged isomers (eq. (23)). And this was the case in all the experiments (Figures 10 and 11).

β-alanine has app. 2 ml greater electrostriction than α-alanine, so that β-alanine should have a somewhat greater compressibility decrement. Nevertheless, there exists no significative difference in the compressibility values for α-alanine and β-alanine. The difference in electrostriction, however, is not great in comparison with the stated accuracy of app. 0.6 ml, and the lack of agreement does not contradict the correctness of the theories of chapter I.

Experimental determination of constant ζ. There exists no possibility of determining ζ from the slope Γ owing to the uncertainty of $\triangle\beta/v$ for low concentrations. The surest way to arrive at this constant is by utilising the formula (26). The difference δ for charged and uncharged isomers was determined graphically at the concentration $v = 0.1$. The different values obtained for ζ from the experiments with amino acids are given in table 14 together with the values for molar volumes and electrostriction according to Cohn and Edsall (33). The reason for the concentration $v = 0.1$ being taken is because in this way the surest value for ζ is obtained. With low concentrations the uncertainty is great for $\triangle\beta/v$ and for high concentrations the formulae derived do not apply. The variation to be found with ζ is due for the greatest part to uncertainty regarding the magnitude of the electrostriction. The mean value $\zeta = 1.1 \cdot 10^{-10}$ cm²/dyne is a probable value.

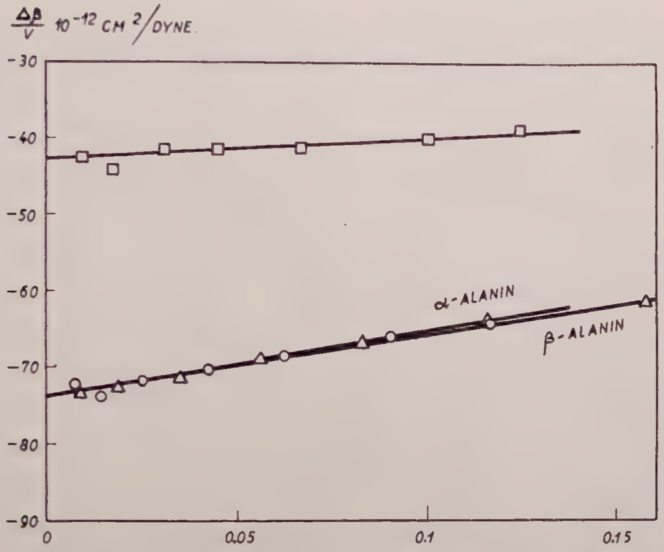

Fig. 11. Total compressibility decrement as a function of fraction by volume of lactamide (□) and its isomers α-alanin (○) and β-alanin (△).

Table 14

Various constants for amino acids and uncharged isomers.
(Φ_0 = apparent molar volume at infinite dilution, $\Phi_{v=0.1}$ = molar volume at $v = 0.1$, E = electrostriction at $v = 0.1$.)

Solute	Φ_0 ml	$\Phi_{v=0.1}$ ml	E ml	δ 10^{-12} cm^2/dyne	ζ 10^{-12} cm^2/dyne
Glycolamide . . .	56.16	56.43			
Glycine	43.20	44.73	11.70	30.3	112
Lactamide	73.51	73.53			
α-alanin	60.61	61.39	12.14	25.2	119
β-alanin	58.73	59.70	13.83	25.3	99
				$\zeta_{\text{mean}} = 110 \approx$ $\approx 1.1 \cdot 10^{-10}$ cm^2/dyne	

Chapter V. General observations on solutions of macromolecules

Though it is of great interest, only slight attention has been directed to the compressibility in solutions of macromolecules. **Herzfeld** (34) derived in a theoretical work a formula for adiabatic compressibility in emulsions. That author bases himself on the emulsified particles being regarded as spheres which scatter the sound waves. This assumption seems to be incorrect when the particles are small in relation to the sound-wave length, which is always the case in measurements such as can be performed in practice. The formula derived was tested experimentally by RANDALL (30) and was found to lead to unreasonable results. From the theoretical point of view it is more probable that the compressibility for suspensions and solutions of macromolecules is an additive function of the product of the compressibility and the concentration of the constituent materials. According to URICK (35) this was found to be the case with a number of suspensions and emulsions, which thus follow the equation (4). From this we may draw the conclusion that in principle macromolecular solutions may be regarded in the same way as the low molecular solutions discussed earlier.

In this connection it is of interest to discuss whether it is possible to compute the compressibility of the macromolecules from the compressibility of the macromolecular solution. For low molecular substances this is not possible, as the compressibility in this case is determined entirely by the intermolecular forces. Thus a low molecular substance has a compressibility of 10^{-10} cm²/dyne, whereas the internal compressibility of the substance's molecules is in the order of magnitude of 10^{-14} cm²/dyne.[1] With macromolecules the problem is quite different, when the limit value 1 is approached for the space ratio and only an insignificant part of the volume fraction of the solute consists of intermolecular spaces. The value that is obtained for the compressibility of the macromolecular substance from the values for solutions of same will then be dependent in higher degree on the compressibility of the molecules themselves than is the case with low molecular substances.

Breaking of the association of water. Just as with low molecular substances the association of water will be broken through surface interaction when macromolecules are dissolved in it. This breaking, however, will be smaller the larger the molecules are. To present this quantitatively the constant B has been studied in figure 12 as a function of the relation between the molar volumes V_1/V_2 for different ratios of the form factors f_2/f_1. From this it is seen that the correction term in equation (12) e.g. for a molar volume ratio of 1 : 1 000 has only one tenth of the value for equal molar volumes. The magnitude of the correction term is diminished still further by the circumstance that only macromolecules which form strong hydrogen bonds association with water are soluble in water, so that $(1-\vartheta)$ assumes a small value.

The theoretical computation of the hydrogen bonding factor ϑ for macromolecules involves certain difficulties. Our knowledge of these molecules is so far not such that it is possible to compute the exact number and nature of the atoms on the molecule surface that are exposed for association with the water. A probable value can nevertheless be obtained, as the constitution of the smaller units of which the macromolecules are built up is known. ϑ should, in fact, have approximately the same value for equal percentages of composition and density with different molecules irrespective of their magnitude and form. This is valid, however, only on condition that the atoms disposed to association are on the average distributed equally in the volume

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[1] According to KITTEL (41) the internal compressibility β_{mole} is of the order of magnitude of l/k, where l is the length of the molecule and k is the force constant. The force constant is given by $k = (2\pi\nu)^2$ where ν is the frequency for fundamental vibration of the molecule and is of the order 1 000 cm^{-1} or $3 \cdot 10^{13}$ c. p. s. and m is the reduced mass of the molecule, this being of the magnitude 10^{-22} g for a light molecule. With these values we get $k = 10^6$ dyne/cm², so that $\beta_{\text{mole}} = 10^{-14}$ cm²/dyne.

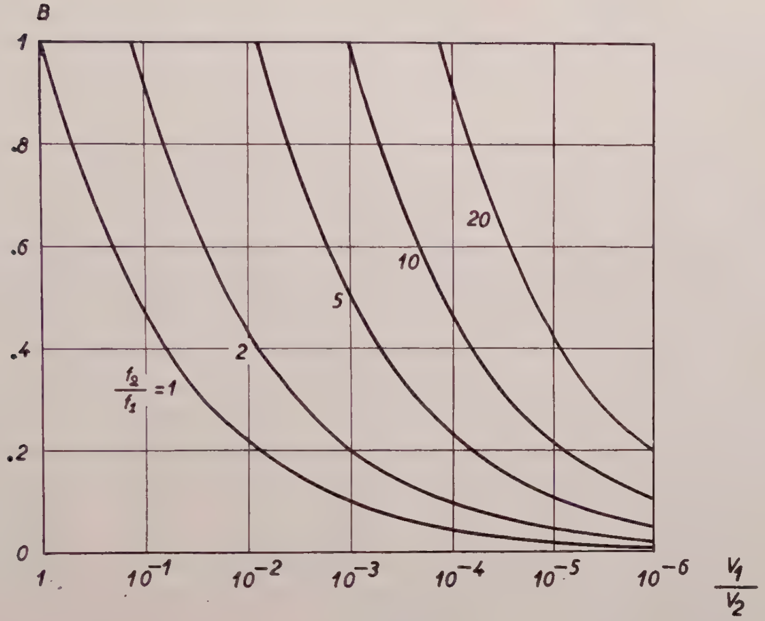

Fig. 12. The constant B as a function of the ratio between the molar volumes of solvent V_1 and solute V_2 at different ratios of the form factors.

of the molecule. If the macromolecules are composed of differing monomers then ϑ can suitably be computed from an imagined medium-sized monomer with a given mean composition. If the monomers contain different atoms disposed to association such as oxygen and nitrogen, there should be taken into account the different dispositions to association of these atoms to the water structure. Failing the exact relation between them and in view of the uncertainty attaching to the computations in general, I have considered it justifiable to make the approximation of assuming the same association tendency to oxygen and nitrogen. The degree of association may be put at 1.7 hydrogen bonds per atom at 20° C in analogy with the computations in Chapter III.

For the low molecular substances, μ is determined experimentally. The possibility of corresponding computation of μ for macromolecular substances is decreased both because the exact concentration is difficult to obtain and because the substance is generally not soluble to high concentrations. Apart from the purely theoretical interest of μ for macromolecules, the exact value is of small significance for the interpretation of the measurement results generally. Thus in the correction term in formulae (12) and (14) μ is included in the term $(1 - v^{\mu})$, and since v is small any error in μ will be without any great influence on the magnitude of the correction term. It is only with extremely small values for μ that any great error can arise.

Electrostriction. When the macromolecules have permanent polar groups, electrostriction will be present as with low molecular substances. The magnitude of the electrostriction is dependent on the number of groups on the molecules. This number can be found by different titration methods. The distance between the groups has less influence as with increasing distance the electrostriction tends to a limit value. For example, this limit value according to Schmidt (36) is app. 20 ml/mol for substances with one $-\mathrm{NH}_3^+$ and one $-\mathrm{COO}^-$ group per molecule.

Chapter VI. Proteins and desoxyribonucleic acid

For the study of the compressibility of protein solutions the substances serum albumin (horse), oxyhaemoglobin (horse) and haemocyanin (Helix pomatia) were selected. These substances are soluble to high concentrations and their other physical and chemical properties have been thoroughly investigated. For these reasons they are well suited for an experiment of general guiding tendency in macromolecules.

Theoretical predictions. As the proteins are polar the formulae (24) and (25) should be suitable for computation of the compressibility in solutions of them. We shall begin by discussing the influence of electrostriction. According to Schmidt (36) and Cohn and Edsall (33) the electrostriction for protein solutions is small and in the order of magnitude of 2 % of the molar volume. For a molecular weight of 10^5, a density of 1.35 and with a concentration of $v = 0.3$ the following value is obtained for the correction term for electrostriction $e\zeta(1-v) = 0.3 \cdot 10^{-12}$ cm²/dyne. The value decreases with lower concentrations. The order of magnitude of the term is thus in these cases small in comparison with the orders of magnitude of compressibility of aqueous solutions $40 \cdot 10^{-12}$ cm²/dyne. For this reason the term will be ignored in these cases, even though this will involve a certain small systematic error of the order of 1 %. This appears preferable as the exact magnitude of the electrostriction for the proteins is uncertain. The formulae (24) and (25) are by ignoring the electrostriction reduced to (12) and (14) respectively.

To make clear the influence on the compressibility due to breaking of the association of the water through surface interaction there will be computed the magnitude of the correction term for this in the formulae (12) and (14). The constant B has been determined for the different proteins by the known values for molecule weight and axial ratio obtained by other experimental methods (Cohn and Edsall) (33) and is given in table 18. The density values employed were obtained by own experiments and agree well with the values given by other experimenters. The value for ϑ has been assumed to be equal for the proteins and was obtained in the manner indicated in chapter V. The average composition of the "monomers" has been assumed to be 2.5 oxygen atoms and 1.5 nitrogen atoms per molecule and to have a molecule weight of 130 and a density of 1.34. These values are a mean of the ordinary amino acids and give $\vartheta = 0.65$, which is a reasonable value for proteins. With the above assumptions, $(1-\vartheta)B\xi$ has been computed and is given in table 18 for the different proteins. The magnitude of the constant μ is troublesome to determine, according to what is stated in chapter V. It is probably of the order of 0.1 if comparison is made with the low molecular substances in chapter III. The value is not critical and $\mu = 0.1$ is assumed to be valid. In tables 15–17 where the experimental results are given there will be found also the term $(1-v^{\mu})(1-\vartheta)B\xi$ for the different concentrations. As may be seen from this, the correction term is small but decreases somewhat with increasing concentration. From this the conclusion may be drawn that if formulae (12) and (14) are applicable then the total compressibility decrement $\triangle \beta / v$ should be relatively constant for different concentrations, though with a suggestion of increase with rising concentration.

Experimental results. As may be seen from the results of the measurements in tables 15–17, the total compressibility decrement $\triangle \beta / v$ for serum albumin and haemoglobin is mainly constant, though with a suggestion of increase towards higher concentrations, in striking agreement with the theoretical expectation. Owing to the greater dispersion in the experimental values and owing to the smaller value of the correction term a corresponding tendency in the compressibility decrement does not appear for haemocyanin. The agreement is best for serum albumin where the experimental errors are small owing to it being possible to attain high concentrations because of the high solubility. There has also been computed the difference between the proteins' and the water's compressibilities $\beta_2 - \beta_1$. This should be constant for the different concentrations and it is so within the experimental limits of error. The values for serum albumin are most convincing and display great constancy. In computation of the mean value for $\beta_2 - \beta_1$ the values for the different concentrations have been given the weight corresponding to their concentration.

For lower concentrations than those given in tables 15–17 the different protein solutions display compressibility values with a certain typical deviation from linearity and this may be explained in principle by the theory developed in chapter I. The observation requires, however, more thorough study before it can be interpreted in terms of intermolecular interaction. Nevertheless, it is worth stating that compressibility measurements appear to be a serviceable auxiliary for the study of intermolecular properties of macromolecules.

Discussion of the compressibility in pure protein substances and its relation to radio frequency spectra. It is possible to obtain the compressibility of the proteins β_2 immediately from the mean value for the difference between the compressibilities of the proteins and the water $\beta_2 - \beta_1$ from the different experiments. As may be seen in table 18 the compressibility has approximately the same value for the different protein substances. The difference falls entirely within the experimental limits of error. As stated in chapter V the value for macromolecular substances' compressibility should be dependent in high degree on the internal compressibility of the molecules, when the intermolecular spaces are small. These spaces must owing to the considerable difference in molecule size be appreciably greater for, e.g. haemoglobin than for haemocyanin. The fact that no difference exists in the compressibility values determined experimentally for haemoglobin and haemocyanin, suggests that this is entirely determined by the internal compressibility of the molecules. This reasoning is valid, of course, only on condition the molecules of the different protein substances have the same compressibility, which is very probable as they have similar physical and chemical properties otherwise.

Assuming β_2 to be considered as equal to the internal compressibility β_{mole} of the protein molecules, then according to LOVE (37) the modulus of elasticity of the molecules can be obtained from the following formula, which gives the relation between the volume compressibility of an isotropic body and its linear modulus of elasticity.

$$E = \frac{3(1 - 2\sigma)}{\beta_{\text{mole}}} \tag{27}$$

Table 15

Experimental values and calculated constants for horse serum albumin in water solution at 20° C.

Concentration g/ml solution	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne	$\Delta\beta/v$	$(1-v^u)\cdot(1-\vartheta)B\xi$	$\beta_2-\beta_1$
0.363	1.6017	1.0997	35.45	−37.3	−0.8	−36.5
0.239	1.5568	1.0619	38.86	−37.2	−1.0	−36.2
0.163	1.5321	1.0416	40.90	−37.5	−1.2	−36.3
0.107	1.5147	1.0273	42.43	−37.7	−1.4	−36.3
0.0704	1.5046	1.0177	43.40	−38.6	−1.7	−36.9
0.0472	1.4979	1.0111	44.08	−38.0	−1.9	−36.1
0.0		0.9982	45.40	Mean value of $\beta_2-\beta_1 = -36.3$		

Table 16

Experimental values and calculated constants for horse oxyhemoglobin in water solution at 20° C.

Concentration g/ml solution	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne	$\Delta\beta/v$	$(1-v^u)\cdot(1-\vartheta)B\xi$	$\beta_2-\beta_1$
0.140	1.5261	1.0331	41.56	−36.6	−1.2	−35.4
0.0977	1.5131	1.0220	42.74	−36.2	−1.4	−34.8
0.0653	1.5046	1.0141	43.56	−37.5	−1.5	−36.0
0.0466	1.4983	1.0104	44.09	−37.4	−1.7	−35.7
0.0318	1.4949	1.0062	44.47	−38.9	−1.9	−37.0
0.0214	1.4919	1.0035	44.77	−39.1	−2.0	−37.1
0.0		0.9982	45.40	Mean value of $\beta_2-\beta_1 = -35.6$		

Table 17

Experimental values and calculated constants for Helix pomatia hemocyanin in water solution at 20° C.

Concentration g/ml solution	Sound velocity 10^5 cm/sec.	Density$_4^{20}$	Compressibility 10^{-12} cm^2/dyne	$\Delta\beta/v$	$(1-v^u)\cdot(1-\vartheta)B\xi$	$\beta_2-\beta_1$
0.155	1.5282	1.0382	41.24	−35.9	−0.25	−35.6
0.0969	1.5123	1.0235	42.72	−36.8	−0.31	−36.5
0.0700	1.5020	1.0164	43.61	−33.8	−0.34	−33.5
0.0497	1.4969	1.0107	44.16	−32.7	−0.37	−32.3
0.0344	1.4946	1.0064	44.48	−34.8	−0.40	−34.4
0.0247	1.4912	1.0042	44.78	−32.1	−0.43	−31.7
0.0		0.9982	45.37	Mean value of $\beta_2-\beta_1 = -34.8$		

Table 18

Different constants for proteins.

Protein	Molecular weight[1]	Density	Axial ratio[2]	B	$(1-\vartheta)\,B\,\xi$ 10^{-12} cm²/dyne	β_2 10^{-12} cm²/dyne
Serum albumin .	70 000	1.36	1 : 5	0.11	−6.6	9.1
Hemoglobin	68 000	1.33	1 : 3.5	0.10	−5.9	9.8
Hemocyanin	$8.9 \cdot 10^6$	1.35	1 : 5	0.02	−1.3	10.6

[1] From sedimentation and diffusion data.

[2] Mean of values from different methods. Here taken as the ratio of short to long side in a parallelepiped with quadratic cross-sectional area.

where σ is Poisson's elasticity ratio. As regards discussion of the numerical value for σ reference is directed to LOVE (37). Here the elasticity ratio for proteins will be estimated as $\sigma = 1/3$. The modulus of elasticity for the protein molecules, which is considered to be equal for the three substances studied, may now be computed as $1.0 \cdot 10^{11}$ dyne/cm².

According to JACOBSON (38), the frequency of the radio frequency absorption spectra of the molecules is determined by the molecular dimensions, the modulus of elasticity E and density ϱ. For longitudinal vibration types, which are those which most easily arise in the molecule, there applies the equation

$$\nu = \frac{i}{2 \cdot l}\left(\frac{E}{\varrho}\right)^{\frac{1}{2}} \tag{28}$$

where ν is the frequency, i an integer and l the length of the molecule. The frequency for fundamental longitudinal vibration can be computed from this for the respective molecules as albumin $1.0 \cdot 10^{11}$, haemoglobin $1.3 \cdot 10^{11}$ and haemocyanin $0.21 \cdot 10^{11}$ c. p. s., equivalent to the wave lengths 0.30, 0.23 and 1.5 cm respectively. The wave lengths correspond to radio waves in the microwave range. Depending upon the uncertainty in the values of the constants in formula (28) the calculated frequencies are only to be regarded as orders of magnitude.

Desoxyribonucleic acid and its enzymatic degradation. Some preliminary experiments have been made on the sodium salt of desoxyribonucleic acid (DNA) prepared according to HAMMARSTEN 1924 (27). These show that DNA in salt-free aqueous solutions has a considerable compressibility decrement ($\triangle \beta / v \approx 110$ cm²/dyne), which is appreciably greater than for all the substances, both high and low molecular, so far studied. As great uncertainty prevails concerning the molecular weight of DNA and the other constants which are required for thorough analysis of the measurement results, I refrain from undertaking this analysis. It may, however, be stated that the main part of the decrement cannot be caused by a breaking of the association of the water through surface interaction, but must be attributed to an electrostriction arising from the action of the polar groups of the DNA molecule. The ratio between electrostriction and real molar volume is of the order of $E/V = 0.3$. This will also mean that the real density of

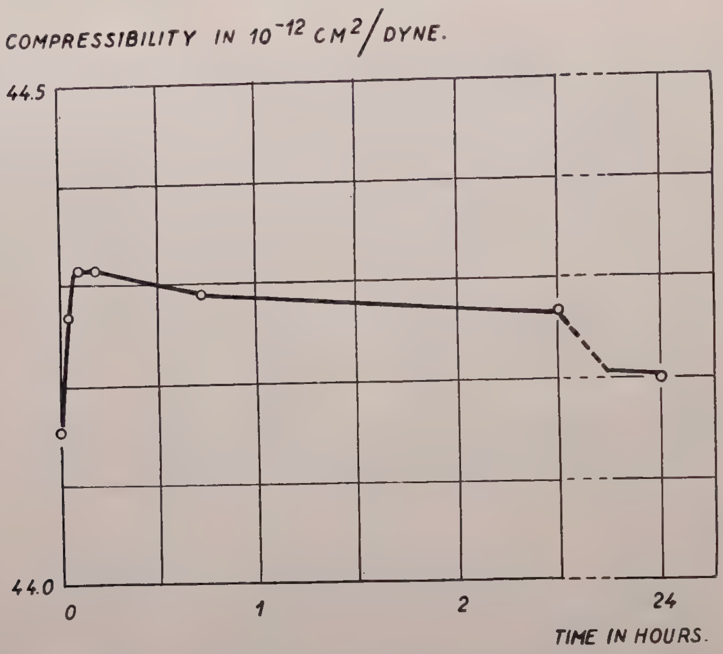

Fig. 13. Compressibility as a function of time during enzymatic degradation of desoxyribonucleic acid by desoxyribonuclease.

DNA is not equal to the apparent 2.0 g/ml, but must have a value approaching that of the proteins, 1.3 g/ml.

To test the possibility of employing compressibility measurements for the study of enzymatic reactions, a preliminary experiment was made with DNA and crystalline desoxyribonuclease. A 1.6 % solution of DNA in 0.03 M veronal-HCl buffer pH 7.5 was digested with 0.001 % desoxyribonuclease in the presence of 0.01 M magnesium sulphate at 20° C. The compressibility varied with the time, as may be seen from figure 13. In computing the compressibility for the first three measurement points the same value for density was used. The magnitude of the density variations in the experiment is small in comparison with the change in sound velocity, so that no great error is caused thereby.

It will be seen from figure 13 that the enzymatic degradation takes place in two phases. There first sets in a rapid increase of the compressibility, which is succeeded by a slow fall. Referring to what was said above about the uncertainty in determining the different constants for DNA, I refrain here from drawing any conclusions from the results. Nevertheless, it is of interest to compare the experiment with the results obtained by Chantrenne, Linderström-Lang and Vandendriessche (39) in their studies of ribonucleic acid with ribonuclease. The initial rapid increase of volume and subsequent slower volume decrease which the above authors found for ribonucleic acid are recalled by the change in compressibility with my experiment. A comparison is also appropriate with the dielectric investigation undertaken by Jungner, Jungner and Allgén (40), with digestion of DNA with crystalline desoxyribonuclease, in which several stages were noted. In the initial stage there occurs a very abrupt fall in the dielectric increment. This fall probably corresponds to the increase of compressibility in my experiment, but the experiments are not directly comparable, owing to difference in experimental conditions.

SUMMARY

Chapter I. A theory has been worked out for the compressibility in solutions. Three cases are treated. Firstly, compressibility in ideal solutions can be an additive property of the product of compressibility and concentration of the substances included. (Equation (4).) As a second possibility, the case has been treated where the solvent is associated with hydrogen bonds and this association is broken when the solute is dissolved. The compressibility alteration for breaking of the association is a function of that part of the solute's molecular surface that comes into contact with the solvent. (Equation (12).) As a third possibility, the case is treated where in addition there arises electrostriction in the solvent. (Equation (24).)

Chapter II. An ultra-sonic interferometer method for determining sound velocities was developed with particular attention to biochemical investigations. The requisite solution volume has been brought down to 4.5 ml. The ultra-sound intensity is extremely low and the power developed in the solution is less than $5 \cdot 10^{-4}$ watt. The relative accuracy is 0.05 %.

Chapter III. To test the value of the formulae (4) and (12) in chapter I the compressibility was measured in seven liquid mixtures of varying concentration. The mean deviation between theoretically computed and experimentally ascertained relative values with homogeneously distributed concentrations varies for the respective mixtures between 0.68 and 1.94 %. The agreement has been regarded as good and the results obtained do not conflict with the validity of the formulae.

Chapter IV. Determination has been made of the compressibility of solutions of varying concentration with the isomers glycine and glycolamide as also α-alanine, β-alanine and lactamide. As the magnitude of the electrostriction in solutions of the amino acides are known the value for the compressibility change factor with electrostriction may be determined as $\zeta = 1.1 \cdot 10^{-10}$ cm²/dyne.

Chapter V. The internal compressibility of macromolecules will determine in higher degree the compressibility of a solution thereof than is the case with low molecular solutes, where the compressibility is entirely governed by intermolecular forces.

The breaking of the association of water through surface interaction will have less influence on the compressibility with macromolecular solutions compared with low molecular solutions. The influence of electrostriction is mainly a function of the number of polar groups on the macromolecule.

Chapter VI. The compressibility of salt-free aqueous solutions of serum albumin, haemoglobin and haemocyanin were measured. The values obtained agree very well with what might be expected from the theoretical computations of chapters I and V. The compressibility for pure protein, which is probably also the compressibility of the protein molecules, is the same for the three substances, the value being $1.0 \cdot 10^{-11}$ cm²/dyne. This value does not differ greatly from the compressibility of many metals.

The frequency for radio frequency spectra of the protein substances investigated may be computed theoretically to the microwave range.

Desoxyribonucleic acid gives a very large compressibility decrement in aqueous solutions, which can only be explained by assuming a considerable electrostriction.

A preliminary experiment with enzymatic degradation of desoxyribonucleic acid with desoxyribonuclease shows that measurements of compressibility may be utilised for the study of enzymatic reactions.

Karolinska Institutet, Biochemical Department, Stockholm.

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On the mechanism of electrophilic aromatic substitution

An investigation by means of the effect of isotopic mass on reaction velocity

By LARS MELANDER

With 9 figures in the text

Contents

I. The development and present state of the theory of aromatic substitution........ 213
  1. Short survey of earlier theories and results........ 213
    1 a. The substitution of hydrogen as a typical reaction of the aromatic molecule... 213
    1 b. The rules of orientation........ 214
    1 c. The development of modern physical organic chemistry........ 215
    1 d. Pictures of the mechanism of aromatic substitution........ 216
  2. The present picture of the electrophilic mechanism........ 219
    2 a. The electronic structure of benzene........ 219
    2 b. The electrophilic mechanism of replacement........ 220
    2 c. Results of competitive substitutions........ 221
    2 d. The qualitative theory of the English school........ 221
    2 e. Attempts at a quantitative treatment........ 223
  3. Problems still unsolved........ 224
    3 a. Difficulties with the current methods........ 224
    3 b. Hypothetical pictures of the mechanism of replacement........ 225
II. Scope and general method of the present investigation........ 225
  4. Different possible mechanisms of the electrophilic substitution and the use of isotopic hydrogen........ 225
    4 a. Reaction rates of isotopic hydrogen........ 225
    4 b. The action of catalysts........ 226
    4 c. Mechanisms to be excluded........ 227
    4 d. The pure S_E2 mechanism........ 227
    4 e. Substitution via an intermediate addition compound........ 228
    4 f. Kinetical possibilities........ 229
    4 g. π-Complex formation........ 231
  5. The arrangement of experiments with isotopic hydrogen........ 231
III. Theoretical part........ 232
  6. The theory of absolute reaction rates and the behaviour of different isotopes........ 232
    6 a. Limitations of the theory........ 232
    6 b. Fundamentals of the theory of absolute reaction rates........ 233
    6 c. The energy surface and isotopic systems........ 234
  7. Application of the theory of absolute reaction rates to the pure S_E2 mechanism.... 234
    7 a. The ratio k_T/k_H........ 235
    7 b. The partition function ratio........ 235

7 c. The zero-point energy factor ... 240
7 d. The activity coefficient ratio ... 242
7 e. Summary of the discussion of k_T/k_H ... 243
7 f. The ratio k'_H/k_H ... 244
8. Application of the theory of absolute reaction rates to the two-step mechanism ... 246
8 a. Relation of the mechanism to other theoretical considerations ... 246
8 b. The specific rate ratios of the different steps ... 247
9. Variation of isotopic concentrations in isotopic reactions ... 248
9 a. General ... 248
9 b. Competition between two or more positions and between molecules ... 249
9 c. Competition only between two kinds of molecules ... 254

IV. Experimental part ... 257
10. The determination of tritium ... 257
10 a. The unit of specific activity ... 257
10 b. The combustion of tritium compounds ... 257
10 c. Critical discussion of the determination of tritium in water ... 258
10 d. The reproducibility of the determination of tritium in organic compounds ... 259
11. The preparation of tritium compounds ... 260
11 a. The preparation of tritium ... 260
11 b. General discussion of preparative methods ... 260
11 c. The Grignard reaction in practice ... 261
11 d. The present preparations by means of the Grignard reaction ... 262
11 e. Investigation of possible exchange in the preparation of tritium hydrocarbons ... 266
11 f. Preparation of benzene-t by exchange ... 266
11 g. The specific activity of the tritium preparations ... 267
11 h. An attempt to prepare nitrobenzene-3-t by reduction of m-nitrophenyldiazonium sulphate ... 268
12. General principles of the substitution experiments ... 269
13. Experiments on nitration ... 270
13 a. Dinitration of benzene ... 270
13 b. Dinitration of toluene ... 271
13 c. Dinitration of bromobenzene ... 273
13 d. Nitration of naphtahalene ... 274
14. Experiments on bromination ... 275
14 a. Bromination with aluminium chloride as catalyst ... 275
14 b. Bromination with ferric bromide as catalyst ... 276
14 c. Bromination with iodine as catalyst ... 277
15. Experiments on sulphonation ... 278
15 a. Sulphonation of benzene ... 278
15 b. Sulphonation of bromobenzene ... 280

V. Discussion of results ... 280
16. Nitration ... 280
16 a. The influence of side-reactions ... 280
16 b. Dinitration of benzene-t ... 281
16 c. Dinitration of toluene-2-t ... 282
16 d. Dinitration of bromobenzene-2-t ... 284
16 e. Nitration of naphthalene-α-t ... 284
16 f. The probable mechanism of nitration ... 284
17. Bromination ... 286
18. Sulphonation ... 287

Summary ... 289
References ... 291

I. The development and present state of the theory of aromatic substitution

1. Short survey of earlier theories and results

As most of the current monographs on physical organic chemistry have chapters dealing with the problem of aromatic substitution and generally also with the more important theories which have now been abandoned, this survey is made rather short. The well-known monographs by W. HÜCKEL (1), A. E. REMICK (2), H. B. WATSON (3), and the chapter on aromatic compounds by L. F. FIESER in Gilman's Organic Chemistry (4) may be mentioned among those works containing historical information. In a paper which appeared in 1924 (5), A. F. HOLLEMAN gave a good historical survey of previous rules and theories.

Throughout the present paper the word "substitution" is used in the more limited sense of replacement of hydrogen.

1 a. The substitution of hydrogen as a typical reaction of the aromatic molecule. By means of certain reagents it is possible to replace the hydrogen atoms of aromatic molecules with other atoms or groups, e.g. halogens, the nitro group, etc. Such reactions generally proceed rather smoothly and without appreciable destruction of the carbon skeleton. By a proper choice of the experimental conditions it is generally possible to make the substitution stop at a fixed point, for instance after the introduction of one or two substituents. This behaviour contrasts with that of the saturated aliphatic compounds, the hydrogen of which is rather difficult to replace. When such a reaction is brought about it does not stop at a definite point, but leads to a mixture of molecules substituted to a different extent. The halogenation of simple paraffins, which takes place at high temperatures or in the presence of light or certain catalysts, may be mentioned as an illustration. The unsaturated aliphatic compounds have an intermediate position, but generally addition is the prevailing characteristic reaction. Cases are known, however, which seem to be closely analogous to the aromatic substitution. These cases have been very important for the discussion of the mechanism of substitution, and there will be many occasions to consider them in the present paper.

The aromatic substitution is of great importance as it makes different derivatives accessible from the hydrocarbon as starting material. In the aliphatic series, on the other hand, the saturated hydrocarbons have been very poor as a starting point for the preparation of their derivatives. Thus it was only comparatively recently that useful methods were developed for the transformation of paraffins into other aliphatic compounds. The unsaturated aliphatic hydrocarbons are much easier to attack, and one of the best methods to manage the conversion of paraffins is to change them into olefins by means of dehydrogenation.

The property common to the aromatic and olefinic hydrocarbons, which makes them more sensitive to chemical action, is the unsaturation. Nowadays it is known that "unsaturation" or the capability of binding additional atoms or ions consists in the possession of more electrons than necessary for the binding of the original constituents. The chemical attack on the olefins seems to begin with a perturbation on these extra electrons. The present paper deals with the behaviour of the corresponding electrons of the aromatic nucleus in its common reactions. Owing to the fact that the application of ordinary methods used in similar cases is difficult here for various reasons, the existing information is rather scarce.

1 b. The rules of orientation. One of the most striking features of the aromatic substitution is the influence of substituents already present in the aromatic molecule. As the experiments deal almost exclusively with benzene and its derivatives, only the rules of substitution in the benzene ring will be treated in the following, although they have analogies in the condensed aromatic systems, e.g. naphthalene.

As to their directing influence on the great majority of substitutions the substituents may be divided into two classes: those directing the entering substituent mainly into the ortho and para positions and those directing it mainly into the meta position. Some examples are given in Table 1: I.

Table 1: I

Directing influence of substituents in benzene.

Directing into ortho and para	Directing into meta
$-CH_3$ (and alkyl in general)	$-NO_2$
halogens	$-SO_3H$
$-OH$	$-COOH$
$-NH_2$ and $-NHCOCH_3$	$-N^+(CH_3)_3$

The orientation rules hold independently of the nature of the entering substituent and the attacking reagent, as well as that of the surrounding medium. Some rare reactions exist, which do not follow the rules, but they are of minor importance.

The laws of orientation are of great importance for the preparative chemistry, since the directing power is generally of such a magnitude that the opposite isomer is formed only in a yield of a few per cent. It is also possible to transform for instance an amino group into a nitro group, thus changing the directing properties of a group in a simple manner. The amino group may also be eliminated rather simply and replaced by hydrogen after its directing power has been used.

The existence of three different derivatives of formula $C_6H_4X_2$ has been known for a long time, it was so already when KÉKULÉ suggested his benzene formula in 1865. Some years later KÖRNER proved the equivalence of the six hydrogen atoms of benzene, and he, GRAEBE, and LADENBURG carried out absolute position determinations for the derivatives. At that time it was also quite evident that all three possible derivatives were not formed in equal amounts, when a monosubstituted benzene molecule was substituted once more. During the period 1875–1900 this fact caused the formulation of several rules, which tried to connect the directing behaviour of the first substituent with its chemical character, e.g. its acidity, the valence of the substituent atom or the atoms of a substituent group etc. In 1902 VORLÄNDER gave the rule that unsaturated groups are meta-directing, and saturated ones orto- and para-directing. This holds rather well in the common cases, as could be seen from the foregoing survey, but is not completely adequate. In fact, it touches upon one but not all of the important features, and naturally in a very superficial manner, as nothing was known at that time of the electronic structure and the real nature of "unsaturation."

In 1895 HOLLEMAN started his important work on the substitution of benzene. Instead of qualitative reasoning he attacked the subject by means of quantitative determinations of the relative amounts of isomerides formed in the substitution of benzene rings, already carrying one or two substituents. These reactions were carried out under well-defined conditions, especially of temperature, and the quantitative determinations had a high degree of accuracy. Determinations of this kind had been completely neglected before, owing to the analytical difficulties.

The importance of these investigations is quite evident from the fact that Holleman's data have been used extensively in tests of different quantitative theories and are still used to-day. Most of the results were collected by HOLLEMAN in his well-known book "Die direkte Einführung von Substituenten in den Benzolkern" (6), but some important results were obtained after it had been published in 1910.

Some important conclusions could be drawn immediately from these results. The reproducibility of the proportions, even under different conditions, made it clear that the isomerides were formed in the ratio of the corresponding substitution rates and generally no subsequent transitions took place. There are a few exceptions, in which the catalyst causes interconversion of the isomerides, for instance bromination of bromobenzene in the presence of aluminium bromide (7) but they are not of general importance.

Another important conclusion was drawn by SCHEFFER (8), who used the temperature dependence of the isomeric proportions to show that the difference in substitution velocity between different positions in the substituted benzene ring was due only to differences in the energy of activation, and that the frequency factors (depending on the entropy of activation) were very nearly the same.

In the first twenty-five years of the present century several theories of orientation in aromatic substitution were given by, among others, VORLÄNDER, LAPWORTH, KERMAC, ROBINSON, and LUCAS. The electron had been adopted as a constituent of the atoms and molecules, and the theories deal with the behaviour of the electrical charges under the influence of different substituents. The theories are described for instance in the books by W. HÜCKEL (1) and REMICK (2) and will neither be described nor discussed here.

It has to be pointed out, however, that the theories now mentioned have many features in common with modern electronic theory of organic chemistry, and that the latter has inherited many of its conceptions from the former. The former were to a large extent founded on postulates, which could not be proved and had no foundation in classical physics. Most of this holds also of the modern theories, but they are built on more extensive empirical knowledge, and they are closely related to quantum mechanics, which is also founded on certain postulates but has proved its reliability beyond any doubt.

1 c. The development of modern physical organic chemistry. The first "modern" picture of the chemical bond was given by Lewis in 1916, who realized that the sharing of electrons, generally a pair of electrons, is the cause of chemical binding, at least of that in ordinary organic compounds. The sharing, however, may be unequal, and the bond is then polar. In the limiting case both electrons belong to only one of the atoms, which corresponds to a purely ionic bond. All these ideas got their final support in the modern quantum mechanics, which was founded about 1925.

Quantum mechanically it is possible to show that the sharing of two electrons between two atoms will generally cause an energy decrease relatively to the separated atoms, which is equivalent to a binding force. Quantum mechanics, however, is, at least with the present methods of calculation, practically unable to give complete solutions of chemical problems other than the simplest, and even in such cases the solutions are approximate. Therefore a kind of semi-empirical calculation has to be used for the treatment of most problems of organic chemistry, and even this treatment is too difficult in many problems.

What is to-day called the "electronic theory of organic chemistry" might be said to consist of a set of rules or mechanisms for the behaviour of the electrons in organic molecules and reactions, especially for the transmission of electrical charge within the molecules. Most of these rules have their foundations in quantum mechanics, but they are at present much more qualitative than quantitative.

There are some discrepancies between what may be called the English and the German schools, e.g. in the case of the so called "alternating effect" of the German school. However, these seem to be disappearing, as for instance in the case mentioned, where the effect seems to be mainly due to more fundamental, unambiguous effects.

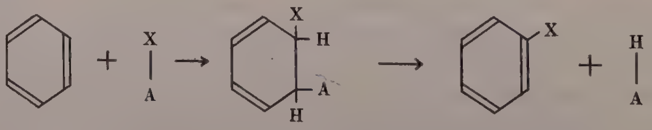
As the theory of the English school is the most well-known at present and seems to be the most self-consistent one, it is used in the present paper. For the German school, reference is conveniently made to the recent monograph by B. EISTERT (9) or to the little older one by E. MÜLLER (10).

The English school originated mainly in the work of R. ROBINSON and C. K. INGOLD. There are some deviations between the branches represented by these names, and that is also the case as to the symbolism. The present state of the system of ROBINSON et al. is evident from a recent monograph by DEWAR (11), and the main features of that of INGOLD are found in various papers, e.g. (12). The monograph by REMICK (2) follows the English school, more specifically the branch of INGOLD.

The picture of the aromatic substitution that is given by the present English school will be described shortly in paragraphs 2 b–2 d.

1 d. Pictures of the mechanism of aromatic substitution. Before the description of the historical development is finished, some earlier pictures of the substitution mechanism are to be mentioned.

For the three main types of substitution, nitration, sulphonation, and halogenation, reaction mechanisms involving additions as the primary step have been suggested. At the time when organic chemistry had not yet adopted ionic reactions as possible, the most natural way of imagining such a reaction seemed to be the addition of two parts of the attacking molecule to one of the double bonds of the Kékulé benzene model:

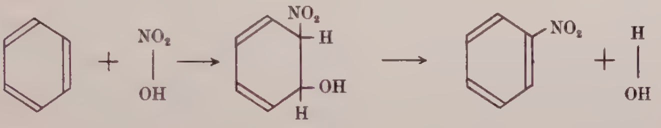


The component of the reagent, which is not to stay in the aromatic molecule, is separated off as the corresponding hydrogen compound. In most cases such a product is actually formed, water in nitration and sulphonation, and hydrogen halide in halogenation. In the case of the simple aromatic molecules the intermediate had never been observed.

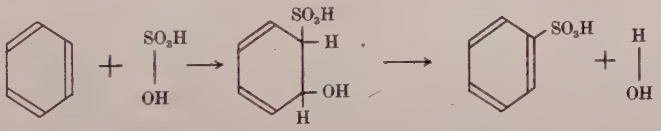
In the case of the olefins the addition product was well known in some cases, e.g. halogenation. It seemed also natural that the corresponding derivative of cyclohexadiene should be unstable and tend to stabilize itself by splitting off either AX (no substitution) or HA (substitution) and pass into the aromatic state. In fact, some aromatic hydrocarbons like phenanthrene, the 9,10-double bond of which is similar to an olefinic one, add bromine and form stable compounds.

The discussion of these problems has been thoroughly reported by W. Hückel (1) and L. F. Fieser (4), where references also are found. A short account will be given here.

Wieland et al. supported the addition hypothesis about 1920 in the case of nitration and sulphonation. The mechanism of the former was supposed to be the following:



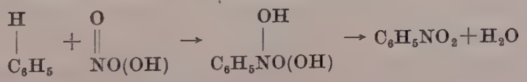
The reason was that compounds analogous to the intermediate had been found with some olefins and even anthracene under nitrating conditions. Much the same was the case with sulphonation, which was supposed to proceed as follows:



About fifteen years later, however, Michael et al. were able to show that neither HNO_3 nor H_2SO_4 reacted with the olefins in the manner assumed. The products were due to other chemical individuals being present in the acids or acid mixtures used, and the pure acids are apt to add themselves to the olefins as $H—ONO_2$ and $H—OSO_3H$, giving esters.

Thus the supposed analogy with the olefins could not be used as a support of the addition hypothesis in the aromatic series, but at the same time the new discoveries did not disprove it, because of the difference in constitution between the two series. Since cyclohexadiene is very unstable the esters could not be supposed to be formed, and the acids might react differently.

Michael suggested another type of addition mechanism, in which benzene is added to an oxygen double bond of the acid, in the case of nitric acid according to:



$$\underset{C_6H_5}{\overset{H}{|}} + \underset{NO(OH)}{\overset{O}{\|}} \rightarrow \underset{C_6H_5NO(OH)}{\overset{OH}{|}} \rightarrow C_6H_5NO_2+H_2O$$

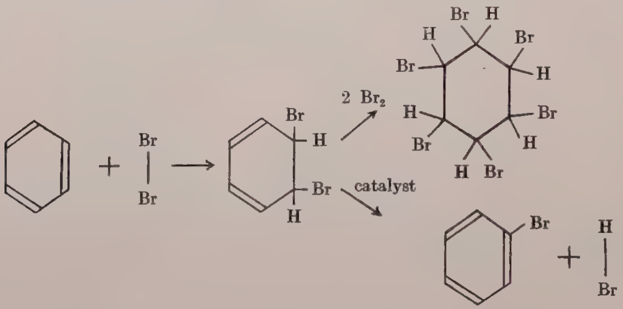
In the case of sulphonation, addition to the anhydride could also occur.

Even the last-mentioned mechanism lacks experimental support, and in the case of sulphonation it is hardly probable since the sulphur-oxygen bonds are almost certainly semipolar and the sulphur has no tendency to enlarge its electron shell.

Recently several investigations have been able to show that the real substituting agents are the cation $(NO_2)^+$ in the case of nitration, and the cation $(SO_3H)^+$, the anhydride SO_3, or its dimer in the case of sulphonation. All three are typical electrophilic reagents with empty electron orbitals.

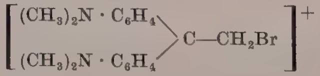
The halogens chlorine and bromine are known to add themselves to the benzene nucleus, forming hexahalogeno cyclohexane. The addition is catalyzed by light. The addition product formed by the capture of only one molecule of bromine is not known. When such catalysts as halides of aluminium, iron, and other metals are present, substitution products are formed without any intermediate being observable.

The two different reactions might be supposed to have a common intermediate of the type indicated above and proceed according to the following scheme:



The olefinic double bond in 1,1-diphenylethylene adds two atoms of bromine, and at higher temperature the 1,1-diphenyl-1,2-dibromoethane loses hydrogen bromide and forms the substitution product of the original olefin, 1,1-diphenyl-2-bromoethylene. This fact seems to support the scheme above, transferred to the olefins.

However, on working with 1,1-di-(p-dimethylaminophenyl)-ethylene, PFEIFFER and WIZINGER (13) were able to isolate an intermediate perbromide, the cation of which,

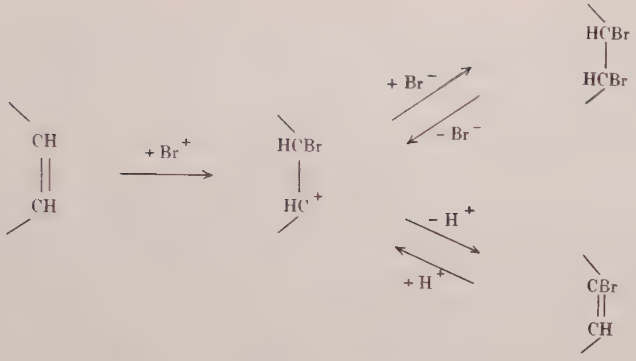


$$\left[\begin{matrix}(CH_3)_2N\cdot C_6H_4\\(CH_3)_2N\cdot C_6H_4\end{matrix}\!\!>C{-}CH_2Br\right]^+$$

also forms a stable perchlorate.

PFEIFFER and WIZINGER proposed that substitutions of olefinic and aromatic substances generally proceed via such intermediates, which are stable enough to be isolated only in certain favourable cases. This would make the action of the complex-forming catalysts understandable, and the authors also proposed analogous schemes for the aromatic nitration and sulphonation.

In the case of bromination of the 9,10-bond in phenanthrene, the results of PRICE (14) seem to support this view. The bond in question is, however, more olefinic than aromatic in type. The most probable general scheme unifying substitution and addition seems to be the following:



It makes it also understandable why the addition product may sometimes give the substitution product without being a real intermediate in the straight-forward formation of the latter. In the case of the benzene ring the intermediate forms a mesomeric system, and thermodynamically only the substitution is possible.

The addition giving hexachlorocyclohexane is accelerated by light and involves an attack by bromine atoms. PRICE (15) assumes also a mechanism for the light-induced addition of bromine to phenanthrene in absence of catalyst analogous to the upper part of the scheme above, but involving atoms instead of ions. The intermediate, then, is a radical and the reaction is of the chain type.

Quite recently the school of ROBERTSON, DE LA MARE et al. have been tending to the opinion that free halogen cations are not necessary in the mechanism of halogenation. The action of the catalyst is proposed to be one of forming favourably polarized compounds, which will work in a manner similar to the simple Br^+.

2. The present picture of the electrophilic mechanism

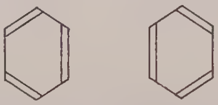
In the following paragraphs some characteristic features of the common aromatic substitution reactions are described in terms of the present physical organic chemistry.

2 a. The electronic structure of benzene. The benzene formula of KÉKULÉ has been generally adopted. It can also be used with safety, if the writer and reader are aware of the limitations of the simple picture and know the physical reality behind it.

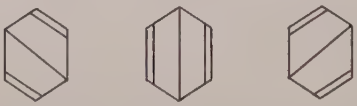
Quantum mechanics has afforded the explanation of the stability of benzene and most of its other properties. The four accessible orbitals for the valence electrons of a carbon atom are the 2 s and the three 2 p orbitals. When six atoms are linked to each other in a ring and each one is also linked to a hydrogen atom, as in benzene, a very stable structure is established in the following way. By linear combination of the s and two of the p orbitals of each carbon atom three orbitals in a trigonal plane structure are formed, corresponding to the σ bonds. The third p orbital will remain on each side of the plane and have its principal direction at right angles to the plane. The whole molecule will be plane, and the six p orbitals outside the plane will interact and form the π orbitals.

The six π-electrons of benzene, which caused so much trouble in the construction of the classical structure, may be regarded as forming a cloud on each side of the ring plane. On the demand of a reagent some or all of them may be localized as σ electrons in the formation of new bonds, as when the benzene nucleus is saturated with hydrogen or chlorine. Substituents generally disturb the symmetry, and the π-electron cloud will not have the same density at all carbon atoms in a benzene derivative. Several more or less refined calculations based on quantum mechanics have been carried out to give account of this influence.

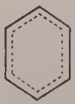
The question of the manner in which the formula of benzene should be written has become one of convenience. In most cases the Kékulé formula is satisfactory. In quantum mechanics calculations are often made on the assumption that the π-electrons interact in a way corresponding to the two possible Kékulé structures



and, to a less extent, corresponding to the three possible Dewar structures



Finally when the mesomerism and the cloud-shape is to be emphasized, the Thiele formula with "partial valences"



is the best picture. In fact, this picture seems to be best in harmony with the present physical conception, and analogous formulae will be used for mesomeric systems in the following.

2 b. The electrophilic mechanism of replacement. Hughes, Ingold et al. have classified replacement reactions according to the distribution of the bond electrons in the displacement process, and this systematization has been generally adopted. Of the two electrons of the original bond, the attacking reagent may take over none, one, or both. The first and third alternatives are named heterolytic mechanisms, the second homolytic. Of the heterolytic processes the first is named nucleophilic, the third electrophilic, according to whether the entering atom or group seeks a nucleus or pair of electrons.

For various reasons, which will not be reviewed here, it has become evident that the common substitution reactions, nitration, halogenation, sulphonation, etc. which all obey the well-known rules of orientation, belong to the electrophilic type. The exceptions from these rules belong to the other two mechanisms.

Already in paragraph 1 d the attacking entities were mentioned. The function of catalysts seems to be to produce the electrophilic species. In some cases they may also act as acceptor for the proton expelled.

The electrophilic reagent will attack the position of highest electronic density, and the directing substituents influence different position differently. There is a very good qualitative correlation between the π-electron density calculated quantum mechanically and the direction of attack.

Before these things are discussed more elaborately, som experimental results should be mentioned.

2 c. Results of competitive substitutions. As mentioned above, HOLLEMAN very accurately determined the relative amounts of the different isomerides formed in the substitution of benzene derivatives. Therefore the directive power of the substituents has been known rather long. However, the influence of the substituents on the reactivity of the nucleus was not revealed by the experiments of HOLLEMAN, i.e. to what extent they activate or deactivate the different positions as compared to unsubstituted benzene. Owing to the complex kinetics of the reactions these proportions have to be determined by means of so called competitive reactions, a method that has been used especially by INGOLD et al. (16).

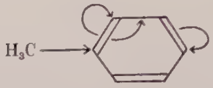
The determinations carried out by HOLLEMAN may be said to be a special kind of competitive reactions. Regardless of the kinetic order of the reaction with respect to the reacting species, the variation in their concentrations during the reaction, etc., the substitution rates in different positions always have the same ratio as their specific rates. All concentrations cancel.

If the problem is to determine the ratio of the specific rates of analogous reactions in analogous molecules, as for instance substitutions in benzene and one of its derivatives, the most convenient and accurate method is to bring an equimolecular mixture of the two organic molecules in reaction with an amount of substituting reagent insufficient for the complete reaction of any of the reactants. As in the preceding case, the amounts formed will be in the same ratio as the specific rates, as far as only small fractions have reacted. If the reaction is more advanced, and if the reactants were not present in exactly equimolecular amounts, the accurate result is obtained by means of a simple formula. (In the last-mentioned kind of competitive reactions the rate of the benzene derivative means its total rate of substitution.)

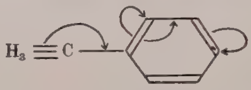
Competitive nitrations of both kinds have been carried out by INGOLD et al. (16). It has been found that meta orientation is always connected with a deactivation of the nucleus as a whole. The meta positions are least deactivated and hence the attacking substituent is directed to these positions. In the case of ortho-para direction things are more complicated, and both activation and deactivation occurs. Thus in toluene the ortho positions react 43 and the para position 55 times more rapidly than if the methyl group were absent. Also the meta positions react more willingly than a position in benzene, actually 3.0 times. The halogens, which are ortho-para directing as well, deactivate all positions, and the corresponding numbers of bromobenzene are 0.037, 0.106 and 0.000. Such results have of course given rise to several attempts at explanation, both of a more qualitative kind and also more quantitative ones.

2 d. The qualitative theory of the English school. Some simple illustrations of the current theoretical treatment according to the English school will be given. Such molecules as have interest for the experimental part of the present paper are chosen.

Toluene is ortho-para directing as stated above. The picture of the mechanism of activation is generally written:

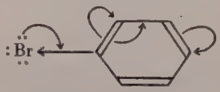


The arrow from the methyl group towards the nucleus indicates that the former is electron-releasing. Since the bond between the methyl group and the nucleus is one between two carbon atoms, there is no difference in electronegativity, but the mesomeric phenyl exerts an attraction. The general inductive (I) effect, operating also in saturated systems, i.e. through σ bonds, increases the electron density also on the ortho atoms, but to a smaller extent, and to a still smaller extent on the meta atoms, etc. By means of the mesomeric effect, indicated by the curved arrows, the negative charge is particularly effectively transferred to the ortho and para positions. Hyperconjugation is certainly also active in this case, since the carbon-hydrogen bonds in the methyl are in some respect similar to a multiple bond and are able to conjugate with the mesomeric system of the nucleus. This could be pictured:



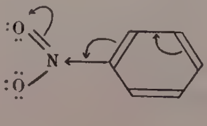
However, since the methyl is electron-releasing, it aquires a positive charge, and generally it is simply characterized as a +I substituent according to the symbolism of INGOLD. All positions for which substitution is possible get their electron density increased, the meta positions to a small extent, the ortho and para positions much more, all in agreement with the empirical data.

Bromobenzene (and other halogenobenzenes) are characterized as —I, +T. By means of the inductive mechanism, bromine, which is more electronegative than carbon, pulls electrons from the ring. The tautomeric mechanism works in the opposite direction, and one of the lone electron pairs of the halogen approaches and interacts with the π-electron system of the ring. This may be illustrated in the following way (the curved arrows show only the direction of the electronic movements caused by the +T action of the bromine):



If the —I effect were stronger than +T, all positions would be deactivated, the ortho and para to a smaller extent than the meta, as the experimental evidence requires. The +T effect is sometimes considered to be limited to a polarizability or "electromeric" effect, which is brought in operation only on the demand of the substituting agent.

Nitrobenzene belongs to the type —I, —T. The nitrogen is more electronegative than carbon and gives rise to the —I effect. The π-electrons of the nitro group conjugate with those of the ring in such a way that electrons are removed from the latter. This is a true mesomeric effect. The picture is:

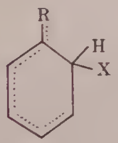


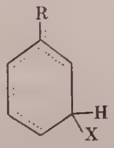
Both effects cause a strong deactivation of the ortho- and para-positions, and the —I effect also deactivation of the meta-positions, but to a smaller extent. Consequently the nitro group is meta-directing, and generally the meta-directing groups may be treated analogously.

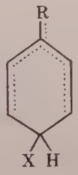
2 e. Attempts at a quantitative treatment. Among the more important theoretical investigations are those by Wheland and Pauling (17), Wheland (18), Seel (19), and Dewar (20).

The first of these papers might be said to be the quantum mechanical interpretation of the electronic mechanisms described in the foregoing paragraph and it deals predominantly with the condition of the unperturbed molecule. In the case of toluene, the influence of the substituent is introduced into the calculations simply as a change in the electron affinity of the ring carbon atom carrying the substituent. In the case of nitrobenzene the problem is treated as one of nine atomic orbitals with ten electrons, etc. The electronic density at each position in the ring is calculated, and with reasonable choice of parameter values it is found that the positions of highest electronic density are just those, where substitution has been found to proceed most readily. In some cases, such as naphthalene, it is necessary to take the polarizability into account. This is done by the addition of an increment to the parameter representing the electron affinity of a certain atom, one at a time. Of the carbon atoms of naphtalene, the unperturbed structure of which was found to possess equal charges on the α- and β-atoms, the α-atoms get the highest electronic density under a polarizing influence, in agreement with the α-direction of nitration.

In the later paper by Wheland the problem is attacked in another manner. The first paper treated the conditions before and in the beginning of the substitution, the second deals with the activated state, the model of which is thought to be structures like







with a complete covalent bond between a ring atom and the entering substituent X. The mesomerism is indicated by "partial valences," as in the paper by Dewar. (Calculations are also carried out for the nucleophilic and radical types of substitution, with the corresponding assumption as to the structure of the complex.) The energies of such structures, carrying original substituents R in different positions, have been calculated. The most rapid substitution should correspond to the activated complex of lowest energy, and satisfactory agreement with the experimental facts has been obtained also in this case.

Later but quite independently the problem was investigated by Seel in the same manner and with the same results.

The calculations by Dewar referred to above follow mainly the last method, but certain refinements have been introduced. The agreement with experimental facts is very good.

The agreement between these quantum mechanical calculations and the experimental reaction rates is qualitative or semiquantitative. An attempt at a quantitative treatment along different lines has been made by RI and EYRING (21). In these calculations the direction of nitration is assumed to be exclusively governed by electrostatic effects. The difference in the free energy of activation between substitution at a certain position in a substituted benzene ring and that in benzene itself is assumed simply to consist of the electrostatic interaction between the charge in the position to be attacked and that of the attacking reagent. In the activated complex, where the total potential energy to be overcome has its maximum, the distance between the entering NO_2^+ ion and the carbon atom is assumed to be 1.6 Å., 10 % larger than the corresponding distance in the final compound. The local dielectric constant is taken as unity. With a knowledge of the relative substitution rates in the different positions of the derivative and benzene, it is possible to calculate the charges in the different positions. By means of simplifying assumptions about the displacement of charge in the molecule it is possible to calculate the complete charge distribution and finally the dipole moment, which is experimentally accessible. A satisfactory agreement has been obtained in such and allied calculations.

KENNER (22) has repeated the calculations for the halogenobenzenes, using a refined method and more extensive empirical data. The agreement between the dipole moments calculated and the experimental ones is very good.

DEWAR (20) has criticized the postulates of such calculations based upon purely electrostatic interactions and pointed out that the success of the calculations is probably due to the general parallelism between polarity and polarizability.

To the quantitative data belong also some kinetical results, but since they have a bearing on the mechanism of the hydrogen replacement, they are more favourably treated in the following.

3. Problems still unsolved

3 a. Difficulties with the current methods. The factors governing the ease and direction of the electrophilic attack on the simple benzene derivatives are rather well known, and the picture seems to involve no serious contradictions or unsolved problems. The mechanism of the replacement of the hydrogen atom, however, is very little known. In the case of substitution at saturated carbon atoms, the existence of optical activity due to asymmetry at the same centre could be used for the study of the mechanism, and considerable progress has been made. There is no corresponding resource in the study of aromatic substitution.

As seen from the quantum mechanical calculation of WHELAND and PAULING and the calculations of RI and EYRING on one hand, and the second quantum mechanical calculations of WHELAND alone on the other, which were all reported in paragraph 2 e, very different suppositions about the activated complex lead to results in agreement with experiments. This is in some respect unfortunate, because nothing is revealed about the detailed mechanism.

The results of kinetical investigations are rather complicated and difficult to interpret. At most it can be hoped that the number of molecules taking part in the process should be settled. Thus the results of HINSHELWOOD et al. (23–25) on aromatic sulphonation with sulphur trioxide in nitrobenzene solution seem to indicate that two molecules of sulphur trioxide are involved in the reaction, but it is impossible as yet to tell if the attacking entity is S_2O_6 or two separate molecules of SO_3, and in the latter case if one of them acts as an acceptor for the proton expelled. Generally the kinetics are much more complicated, and it is often necessary to assume more than one type of reaction proceeding at the same time and leading to the same result, e.g. the results on bromination by ROBERTSON et al. (26, 27). In some cases of nitration, the formation of the attacking entity will determine the over-all velocity of the reaction, and nothing is revealed about the attack itself. These things are evident from several investigations by INGOLD et al., e.g. (28).

3 b. Hypothetical pictures of the mechanism of replacement. Several more or less vague expressions as "double decomposition" or "exchange process" have been used for the replacement reaction. More alternatives exist for a replacement in a substance like benzene than for a saturated compound, owing to the presence of π-electrons in the former. This is seen from the models of the activated complex in paragraph 2 e. These possibilities will be discussed thoroughly in the following. First some hypothetical ideas will be reported.

In connexion with a discussion of the Walden inversion INGOLD et al. (29) suggest a similar mechanism for the aromatic substitution. The reagent attacks from a direction lateral to the plane of the ring, and in the transition state the entering and leaving entities lie in a plane perpendicular to that of the ring.

WATERS (30) seems to be of mainly the same opinion and points out the analogy between such transition states and quinones, although he does not seem to consider the complex as a stable intermediate. The position, the activated complex of which corresponds to the most stable quinone, should be substituted most easily, and this is also shown to be the case.

From the comparison with the quinones the step is very short to the activated complexes of quinonoid structure discussed by WHELAND and SEEL (paragraph 2 e), and from these, in turn, to a reaction proceeding via a more or less stable intermediate of the type suggested by PFEIFFER and WIZINGER (13). LANTZ (31) and BADDELEY, HOLT, and KENNER (32) have proposed such an intermediate in the case of sulphonation and desulphonation.

II. Scope and general method of the present investigation

4. Different possible mechanisms of electrophilic substitution and the use of isotopic hydrogen

The problem of the influence of isotopic mass on reaction velocity will be thoroughly discussed in part III of the present paper. In order to form a foundation of the discussion of the possible mechanisms and their unravelling by means of isotopic hydrogen, which forms the scope of the investigation, some general aspects will already be presented at this point.

4 a. Reaction rates of isotopic hydrogen. There is no general and simple rule for the relative kinetical behaviour of isotopes. Very often the heavy isotopes of hydrogen, ^{2}H, deuterium, and ^{3}H, tritium, are found to react slower than the common, light one, ^{1}H, which is called protium when a distinction is necessary. However, reactions exist, in which a heavy isotope reacts more rapidly than a light one. As will be seen in part III, many different factors influence the rate ratio. Generally the relative magnitudes of the zero-point energies of the initial material and the transition state are most important. In a case where the hydrogen, from being firmly bound by a covalent bond, is loosened and transferred to another bond, the zero-point energy of the initial bond predominates, and the heavy isotopes will react slower than protium.

Some experiments, which are closely analogous to the problems treated here, have been carried out by WYNNE-JONES (33). He studied the isotopic influence on the neutralization of the pseudo-acid nitroethane in heavy water, the slow step of which is the loosening of a hydrogen cation from carbon,

$$CH_3 \cdot CH_2 \cdot NO_2 + OH^- \rightarrow CH_3 \cdot CH^- \cdot NO_2 + H_2O$$

He found that a deuteron was loosened from the methylene carbon with only about a tenth of the velocity of a proton, other things being equal.

In the neutralization of nitroethane in heavy water the deuteroxyl ion acts as an acceptor. In a spontaneous fission of a similar bond with no acceptor present in the activated complex, the zero-point energy of the latter is likely to be lower and the difference in reaction rates still more pronounced.

4 b. The action of catalysts. The action of the catalysts is not completely known, but as already mentioned, it will mainly produce the attacking entity and also sometimes be an acceptor for the proton expelled. In the cases of nitration and sulphonation it is somewhat improper to speak of the sulphuric acid as a catalyst, for it is also the solvent.

In the case of nitration the strong sulphuric acid promotes the formation of nitronium ion from the nitric acid (34–36)

$$HONO_2 + H^+ \rightarrow [H_2O \cdot NO_2]^+ \rightarrow NO_2^+ + H_2O$$

BENNETT et al. (37) claim that sulphuric acid itself and some allied species act as acceptors for H^+, but INGOLD et al. (38) prefer to explain the effect observed in another way.

The sulphonating agent in sulphuric acid and oleum is SO_3H^+ according to HINSHELWOOD et al. (39) and BRAND and RUTHERFORD (40), although sulphur trioxide in other solvents is well known as a sulphonating agent. Evidence exists for the importance of an acceptor. Thus the second molecule of sulphur trioxide in the reactions with this reagent investigated by HINSHELWOOD et al. (23–25) and the solvent nitrobenzene in the experiments with sulphuric acid (39) may operate in such a manner.

Aqueous sulphuric acid is known to cause hydrogen exchange, which is more rapid than the sulphonation if the acid is not too concentrated (41). This interaction is of another type and involves simply a substitution of hydrogen by hydrogen cations. As shown by experiments to be described later, the exchange is too slow to be of importance under nitrating and sulphonating conditions.

In bromination the metal halide catalysts are supposed to form bromine cations:

$$Br_2 + FeBr_3 \rightarrow [FeBr_4]^- + Br^+$$

Iodine as a catalyst is more likely to form favourably polarized species with the bromine (27). Hydrogen exchange caused by aluminium chloride (42) is probably of the same kind as that with sulphuric acid, since $HAlCl_4$ is a strong acid.

Another kind of interaction between catalysts such as aluminium chloride and the aromatic molecules is indicated by the fact that substituents as alkyl groups or bromine may undergo exchange (43). These reactions are very slow under the conditions of the experiments.

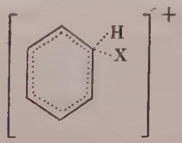
There seems to be no reason to ascribe any other action to the catalytic substances in the substitution reactions than that of forming the electrophilic reagent and in some cases accepting the proton expelled.

4 c. Mechanisms to be excluded. The fact that the hydrogen exchange between the aromatic nucleus and the nitrating or sulphonating acid medium is slower than the substitutions excludes a mechanism of the S_E1 type. If there exists a dissociation equilibrium of benzene, the dissociation is too slow to form a basis for the substitutions. DEWAR (11 a) gives arguments why the phenyl anion should be highly unstable.

A mechanism like the one, which is assumed for aliphatic halogenation does not seem very probable. The attack on the paraffin begins with the pulling off of the hydrogen atom by a halogen atom, formed by photodissociation of a halogen molecule or by a subsequent step of a reaction chain. Since strong evidence supports the picture of a cationoid attack and light has no influence on nuclear halogenation, the only possibility would be that the cation pulls off a hydrogen anion, leaving a phenyl cation that reacts with an anion and forms the final product. Another related possibility is the following. It is known that aluminium chloride is able to transfer halogen atoms and alkyl groups attached to aromatic nuclei, both within and between aromatic nuclei. The same could possibly happen to hydrogen, and a substituent take its place.

The latter possibilities have been rather little discussed and do not seem very probable. They are excluded in such cases where heavy hydrogen is found to be substituted with the same velocity as protium. They will not be discussed in the following.

4 d. The pure S_E2 mechanism. The reactions of type 2 are single-step replacements. In this alternative the π-electrons of the benzene ring are supposed not to take part in the formation of σ bonds, though they are more or less polarized by the positive charge. The activated complex is written

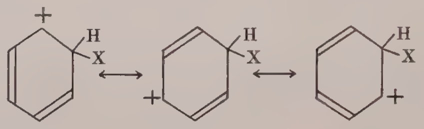


where X denotes the entering substituent. (Sometimes X brings no charge and correspondingly the intermediate will be uncharged.) The replacement takes place at an sp^2-hybridization bond, which is directed outwards from the ring. No inversion, as in aliphatic replacement reactions, is possible, and this fact will increase the potential energy. No conjugation between the π-electron system and the sp^2-bond is possible. This seems to be the same picture as suggested by INGOLD et al. (29), cf. paragraph 3 b.

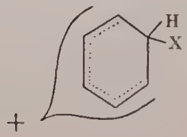
In connexion with a discussion of the reaction of carbonium ions with aromatic compounds, HAMMETT (44) considers a displacement in one step as most probable, because it "avoids the necessity of overcoming the resonance energy of the aromatic substance." He writes the reaction with a base accepting the proton.

The most important feature of the single step mechanism for the present investigation is that the rate of substitution must be dependent on the mass of the replaced hydrogen. The mechanism is possible only in such cases where a heavy isotope is found to react slower than protium.

4 e. Substitution via an intermediate addition compound. Another possibility, which might be more favourable energetically, is that two of the π-electrons are used for the addition of the entering cation, thus forming a more or less stable intermediate, which will subsequently expel the proton. The intermediate could be written:



or



(In some cases X will bring no charge and correspondingly the intermediate will be uncharged as a whole.)

In the former formula all the more important classical structures, between which mesomerism exists, have been given. The latter formula tries to visualize the mesomerism in a single picture. The electronic configuration of the intermediate may be described in words in the following manner. Both the hydrogen atom and the entering group are connected with the carbon atom by complete electron pair bonds, and the latter atom, then, is surrounded by four bonds of the sp³ type. Two of the original π-electrons have been used for the formation of the fourth bond, and the others form a sort of a pentadienate cation system. There may be some conjugation between the latter and the C–H bond. Fig. 4: 1 tries to visualize the intermediate as seen from two different directions. The dashed line gives an idea of the shape of the π-electron cloud.

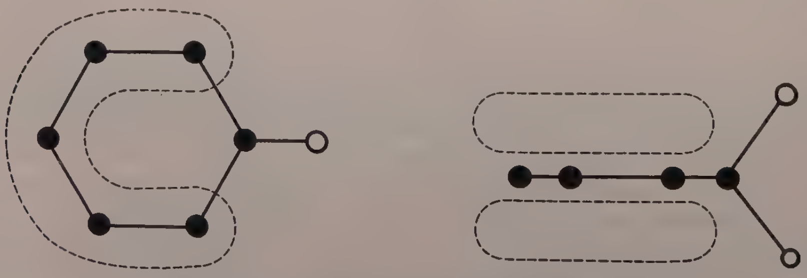


Fig. 4: 1. Hypothetical substitution intermediate.

It should be pointed out that the two structures discussed here and in paragraph 4 d are related and in fact constitute two opposite limiting structures. In the present one some conjugation might occur between the C–H tetrahedral bond and the pentadienate cation structure. If one electron from this bond and one from the C–X bond leave these bonds and join the other four, the common aromatic sextet and the activated complex of paragraph 4 d are formed. The former structure is likely to possess some stability towards a loss of X^+ and H^+, both of which are held by electron pair bonds. The latter structure, however, has no stability towards the loss of X^+ or H^+; it has the character of an activated complex. (It is not supposed that there is appreciable resonance between these structures.)

As is evident from part I of the present paper the idea of an intermediate with the structure just described in this paragraph originated from PFEIFFER and WIZINGER, and it has been supported explicitly by some and implicitly by many authors. Most of them, however, have not been inclined to consider it to be so stable as is done in this paper.

The most prominent characteristic of the intermediate is that the hydrogen is assumed not to be loosened in the activated complex of its formation. This means that the isotopic mass of the hydrogen is assumed to have no effect on its rate of formation.

According to the views put forward in paragraph 8 a, the carbon-hydrogen bond will be of about the same strength in the intermediate as in the initial molecule. That being the case the equilibrium and the reversed reaction will also be unaffected by differences in the hydrogen mass.

4 f. Kinetical possibilities. The observable macroscopic kinetics of substitution reactions may differ from the true kinetics of the substitution step, as found by INGOLD et al. (28, 35). Since only competitive experiments occur in the present investigation, the rate of production of the attacking reagent is of little interest, and only the kinetics of the substitution step itself will be studied in the following. There is no evidence against but much for the natural assumption that the attack is of the first order with respect to the aromatic molecule.

The total reaction may be considered to be irreversible. That this holds has been proved for the nitration and sulphonation reactions by the fact that no or very small hydrogen exchange takes place with the substituting medium. In the case of bromination the hydrogen bromide leaves the system continuously. In the case of a step-wise reaction irreversibility of only the first step of the forward reaction may hardly be the explanation, for if the second step could be reversed with a velocity comparable to that of the forward velocity of the same step, it is hard to understand why the intermediate should not have been observed.

The pure S_E2 replacement may be written in a general manner as

$$A + B + C + \cdots \rightarrow Y + Z + \cdots$$

A denotes the aromatic molecule, and B, C, . . . the attacking entities (some of which may be of the same kind), a base to accept the proton, etc. If Y denotes the final substitution product, the rate of the reaction could be written

$$d[Y]/dt = k[A][B][C]\ldots, \tag{4:1}$$

where k is the specific rate.

The mechanism with the formation of an intermediate requires a more complicated rate expression. The generalized reaction may be written

$$A + B + C \cdots \underset{v_2}{\overset{v_1}{\rightleftharpoons}} M + N + \cdots ; \; M + P + Q \cdots \xrightarrow{v_3} Y + Z + \cdots$$

where M denotes the intermediate, and the rates are given at the arrows. (The specific rates will be indexed correspondingly.)

Very probably it is possible to use the method of the stationary state, and the two limiting cases where the first or the second step is rate-determining, will be studied.

If $v_2 \ll v_3$, the first step is rate-determining and

$$d[Y] / dt = v_3 = v_1 = k_1 [A] [B] [C] \ldots \tag{4: 2}$$

This is formally the same expression as for the pure $\mathrm{S_E}2$ mechanism, but k_1 is the specific rate for a reaction, in which the intermediate and not the product is formed.

If the second step is rate-determining, $v_3 \ll v_2$, and the intermediate will be in equilibrium with the starting material. The equilibrium constant is $k_1/k_2 = K_{12}$ and

$$[M] = K_{12} \frac{[A] [B[[C] \ldots}{[N] \ldots} \tag{4: 3}$$

The overall rate is

$$d[Y]/dt = v_3 = k_3 [M] [P] [Q] \ldots = K_{12} k_3 \frac{[A] [B] [C] \ldots [P] [Q] \ldots}{[N] \ldots} \tag{4: 4}$$

Thus with the second step determining the rate, the overall velocity is proportional to both the equilibrium constant of the first step and the specific rate of the second.

Since k_2 and k_3 in some respects correspond to each other as being rate constants for the loss of electrophilic pieces at the same atom, it is very probable that they are similarly influenced by a substituent in the ring. Thus the difference in substitution rate for different positions of a benzene derivative should be caused mainly by a difference in k_1 even if the second step is rate-determining.

Thus it is impossible or at least very difficult to get information about the course of the reaction from common kinetical data, especially since the species N probably either does not exist or at least cancels from the rate expression and will not give rise to retardation (e.g. if $B = C = N$).

There seems, however, to exist a possibility of getting more detailed information in the use of isotopic hydrogen.

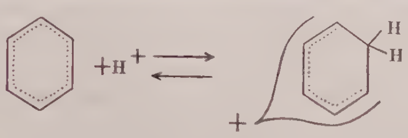
In the single-step mechanism the rate will depend on the mass of the hydrogen, and a heavy isotope will be substituted slower than protium.

In the two-step mechanism, the constant k_1 should be independent of the hydrogen mass, as well as the equilibrium constant K_{12}, cf. paragraph 8 a. k_3, however, is the specific rate of a hydrogen loss and should decrease with increasing mass. Thus the reaction would proceed with the same velocity for different isotopes if the first step is rate-determining, but with a smaller velocity for heavy isotopes if the second step is so.

To sum up, if a substitution is proved to have the same velocity for all isotopes, a stepwise reaction with a rate-determining first step seems to be the only explanation. If a heavy isotope is found to be substituted slower than protium, the reaction might be of the pure S_E2 type or stepwise with the second step rate-determining. These facts form the basis of the present investigation.

The theoretical foundation of the influence of isotopic mass on reaction velocity will be discussed in part III.

A special case of some interest is the hydrogen exchange, taking place under the influence of strong donors of hydrogen nuclei, e.g. sulphuric acid. It is supposed (45, 46) to take place via a mechanism analogous to the common substitutions. In the absence of isotopic hydrogen the reaction may be written as a dynamic equilibrium,



the point of gravity of which is situated far to the left. The specific rates k_2 and k_3 are here identical.

4 g. π-Complex formation. Recently DEWAR (11, 47) has proposed a "π-complex"-theory of the cationoid substitution. The rate-determining step is thought to consist of the formation of a dative bond between the aromatic molecule and the cation by means of the π-electrons of the former and the empty orbitals of the latter. For the transition state of the actual replacement the same possibilities as those above exist, and the heights of the corresponding potential barriers at the different positions will determine the orientation in substituted benzenes. Nothing in the discussion on the application of isotopic hydrogen has therefore to be changed.

It seems DEWAR assumes only one kind of π-complex and that the orientation will be determined only when the π-complex reacts further. The following points of view, however, seem to be worth consideration. The benzene nucleus is rather large, and the π-electron cloud not uniform in the derivatives. It is not very probable that the cation occupies a central position in the benzene ring, where the electron density is not very high. It might be moving around the ring, but will be found most frequently in the positions of highest electron density in substituted benzenes, i.e. at the carbon atoms, where the probability of substitution is highest. The difference in purely coulombic interaction between an ion with unit charge and different carbon atoms (the quantity $\varepsilon_y \varepsilon_n / rD$ in the calculations of RI and EYRING (21)) at a distance of 1.6 Å is sometimes as high as 4 kcal/mol. Hence it seems rather natural to suppose that a movement between positions in the neighbourhood of different carbon atoms should be accompanied by considerable activation energies. It will not be surprising to find π-complexes with the attacking cation firmly bound to a given carbon atom. The difference between such a structure and that of the intermediate described in paragraph 4 e would be one of electron distribution only.

In the case of benzene itself, the entering group may move around the ring almost freely.

5. The arrangement of experiments with isotopic hydrogen

The most sensible method of comparing the substitution velocities of hydrogen isotopes would be to arrange competitive reactions as those of INGOLD et al. between molecules containing the different isotopic species, e.g. between hexadeuterobenzene and benzene. This would require the preparation of macroscopic amounts of the initial substance containing only heavy hydrogen at least in all positions accessible for substitution. In such a preparative work the simplest way would be to prepare the completely deuterated compounds by means of isotopic exchange, which, however, makes the use of large amounts of deuterium necessary.

In this laboratory tritium was accessible, but of course only in radiochemical quantities. Therefore another, less accurate method was followed, the accuracy of which, however, is sufficient for the discrimination between the kinetical possibilities even if the rate ratio cannot be determined with precision.

When unweighable amounts of isotopic hydrogen are used, it is impossible to prepare molecules containing more than one heavy atom each. Only in such experiments, where a single position is substituted, is it possible to arrange a competition analogous to that of INGOLD, in this case between the faint amounts of tritium compound and the bulk of protium compound. Some substitutions of monosubstituted benzenes are directed almost exclusively into para position, and two experiments of that kind have been carried out.

Generally more than one position will come into question for substitution, and when monotriterated molecules are used, competition occurs both within the molecules containing tritium and between them and the bulk, containing no tritium at all. For reasons that will be evident from part III it is necessary in this case to make the chemical conversion as complete as possible. Determinations of the tritium content of the initial substance and the derivative together with the yield gives information about the relative rates of substitution, but the accuracy is much smaller than in the previous case. It decreases rapidly with decreasing yield in the conversion, and it decreases also with increasing number of competing positions in the molecule.

A quantitative treatment of the different possibilities will be given in part III.

III. Theoretical part

6. The theory of absolute reaction rates and the behaviour of different isotopes

The kinetical behaviour of different isotopes is best understood in the light of the theory of absolute reaction rates (48). The main features of this theory will be given first, in order to form the basis for the discussion of the influence of atomic mass. BIGELEISEN (49, 50) has discussed the same problem recently. For convenience a slightly different way will be followed here, and its relation to that of BIGELEISEN will be given.

6 a. Limitations of the theory. Only in certain simple cases, in which the potential energy surface is known in detail, and all the different modes of vibration are known for the activated complex and the reactants, is it possible to give an exact treatment. Generally, however, at least the energy surface is unknown and consequently most data about the activated complex. The latter is the case in the present problem, and much of the discussion will have reference to a purely hypothetical activated complex. Accordingly it is only possible to arrive at probable, not certain results. It is necessary that it should be so in the present case, for the investigation aims at an increased knowledge of the reaction path. Only a quantum mechanical treatment of the hypothetical activated complex would be able to improve the predictions, but even such results would suffer from a lack of exactness.

As regards the initial state, the energy levels of ordinary benzene and its deuterium derivatives are rather well known owing to the extensive work by INGOLD et al. (51, 52). Benzene is chosen as representative for the aromatics used in the experiments, and it is believed that the differences between the individual aromatics do not change the fundamental facts that will be discussed. Thus, for instance it is well known that the C–H stretching frequency 3000 cm^{-1} is observed so generally with the aromatics that it may be regarded as characteristic.

6 b. Fundamentals of the theory of absolute reaction rates. The fundamental postulate of the theory of absolute reaction rates, is that the "activated complex", i.e. the configuration of highest potential energy, which the reactants have to pass in order to form the products, may be regarded mainly as an ordinary molecule thermodynamically, which is in equilibrium with the reactants. There is, however, one important difference. The complex might be said to be unstable towards one of its modes of vibration, for the corresponding movement will lead to decomposition into the products. Most often this is formally treated by the introduction of a fourth translational degree of freedom and correspondingly one of the vibrational degrees of freedom is lost.

It is supposed that the equilibrium between the reactants and the activated complex is maintained by so rapid a mechanism that the decomposition into the products does not disturb the former. Then it will be possible to calculate the concentration of the activated complex by the methods of statistical thermodynamics if the energy levels of the reactants and the activated complex itself are known. When the complex is treated as outlined above, it is found that, of all activated complexes present, those crossing the barrier in unit time constitute a fraction, which has a universal value for all reactions. The rate of reaction may be written:

$$\text{rate of reaction} = c_{\ddagger} \frac{\boldsymbol{k} T}{h} \varkappa$$

where $c_{\ddagger}$ is the concentration of the activated complex and $\boldsymbol{k}T/h$ an universal frequency factor, composed of Boltzmann's constant, the absolute temperature and Planck's constant. $\varkappa$ is introduced as the fraction of activated complexes leading to reaction (some may return to the initial state).

In a bimolecular reaction, the activation equilibrium of which may be written

$$A + B \rightleftarrows (AB)_{\ddagger},$$

the concentration of the activated complex, regarded as above, may be written

$$c_{\ddagger} = K_{\ddagger} c_A c_B \frac{\alpha_A \alpha_B}{\alpha_{\ddagger}} \tag{6: 1}$$

where $K_{\ddagger}$ is the thermodynamic equilibrium constant in concentration units, c_A and c_B the concentrations of the reactants and the α's activity coefficients. Hence

$$\text{rate of reaction} = \varkappa \frac{\boldsymbol{k} T}{h} K_{\ddagger} \frac{\alpha_A \alpha_B}{\alpha_{\ddagger}} c_A c_B \tag{6: 2}$$

The specific rate k is (according to its definition)

$$k = \varkappa \frac{\boldsymbol{k} T}{h} K_{\ddagger} \frac{a_A\, a_B}{a_{\ddagger}} \tag{6: 3}$$

The partition functions for ideal gaseous conditions may be used to express the equilibrium constant, and the activity coefficients have then to be chosen accordingly. If the energy for each molecular species is referred to the lowest level as zero and the corresponding partition functions for unit volume are denoted by F, the specific rate may be written

$$k = \varkappa \frac{\boldsymbol{k} T}{h} \cdot \frac{F_{\ddagger}}{F_A\, F_B}\, e^{-\frac{E_0}{RT}} \frac{a_A\, a_B}{a_{\ddagger}} \tag{6: 4}$$

where E_0 is the difference in energy per mole between the zero-level of the activated complex and that of the reactants, and R the gas constant.

It should be noted that $F_{\ddagger}$ refers to an activated complex which from the statistical point of view differs from a normal molecule only in having one mode of vibration less than a corresponding normal molecule, as outlined above.

The expression for the specific rate does not allow for tunneling through the energy barrier. With a knowledge of the shape of the barrier it is possible to calculate also this effect. Generally it is possible to neglect it, and this will be done in the following discussion.

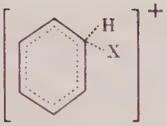
6 c. The energy surface and isotopic systems. Molecules, which have the same chemical composition, but the corresponding atoms (one or more) of which differ in mass, are generally called "isotopic molecules." The term "isotopic reaction" is used for analogous reactions of isotopic molecules.

The energy surface, which has been mentioned already several times, shows the potential energy as a function of the configuration of the nuclei of the system. With the help of some restrictions on the type of configuration two parameters as independent variables are sufficient in simple cases, and three dimensions suffice for the geometrical representation. Differences in nuclear mass will influence the potential energy very little, just as it influences the electronic wave function of a single atom only by a slight change in the reduced mass. Thus isotopic systems have energy surfaces which are practically identical and will be regarded so in the following. Another expression for the same thing is to state that the force constants within isotopic systems are the same.

The differences between isotopic systems arise when the inertia of the atoms comes into play, especially in the vibrations. All this will be evident from the following discussion of the reactions of interest for the present investigation.

7. Application of the theory of absolute reaction rates to the pure S_E2 mechanism

The single step mechanism will have an activated complex of the type



k_T, k_H, *and* k'_H will be used to denote respectively the specific rates of substitution of the tritium atom in benzene-t, a protium atom in the same molecule, and a protium atom in hexaprotiumbenzene.

7 a. The ratio k_T/k_H. According to equation (6: 4) the ratio k_T/k_H becomes after cancellation

$$\frac{k_T}{k_H} = \frac{\varkappa_T}{\varkappa_H} \cdot \frac{F_{\ddagger T}}{F_{\ddagger H}} e^{-\frac{E_{0T} - E_{0H}}{RT}} \frac{a_{\ddagger H}}{a_{\ddagger T}} \tag{7: 1}$$

where the indices T and H denote the isotope being substituted.

The transmission coefficient depends mainly on the shape of the energy surface and the ratio $\frac{\varkappa_T}{\varkappa_H}$ cannot be expected to deviate appreciably from unity (49, 53).

The other three ratio factors will be discussed separately.

7 b. The partition function ratio. The partition function of the activated complex will consist of factors for nuclear spin and electronic degeneracy, and for translational, rotational, and vibrational energy. (The electronic factor is a mere degeneracy factor, as the distance to the first excited electronic level is very large. The change in purely electronic energy for the transition in benzene is as high as 4.88 electron-volts (54) or 113,000 cal/mol, and $e^{-113{,}000/RT} = e^{-160} \approx 10^{-70}$ at 350° K. It is evident that all molecules are in their electronic ground state.)

Since the two complete expressions for k_T and k_H involve ratios of partition functions corresponding to chemical equilibria, the nuclear spin factors disappear by cancellation already in these individual expressions.

The electronic degeneracy will be the same for the two isotopic configurations and cancels in the ratio $\frac{k_T}{k_H}$.

In these approximate calculations it is of course accurate enough to consider the different forms of internal energy independent.

The translational partition function per unit volume has the form

$$f_{\mathrm{tr}}/v = \frac{(2\pi M k T)^{\frac{3}{2}}}{h^3} \tag{7: 2}$$

where M denotes the total mass, and the other symbols have their current meaning. The masses of the two activated complexes are the same. Thus the translational factors of $F_{\ddagger T}$ and $F_{\ddagger H}$ cancel.

The rotational partition function has the form

$$f_{\mathrm{rot}} = \frac{8\pi^2 (8\pi^3 A B C)^{\frac{1}{2}} (k T)^{\frac{3}{2}}}{\sigma h^3} \tag{7: 3}$$

A, B, and C are the principal moments of inertia and σ the symmetry number. Often the nuclear spin and electronic degeneracy factors are included in the rotational partition function, but, as stated above, they may be omitted in this case.

The symmetry numbers for both activated complexes are 1. Thus the rotational partition functions will contribute the factor

$$\left(\frac{A_T B_T C_T}{A_H B_H C_H}\right)^{\frac{1}{2}}$$

to the ratio k_T/k_H.

The vibrational partition function has the form (ν = frequency):

$$f_{\text{vib}} = \prod_i (1 - e^{-h\nu_i/kT})^{-1} \tag{7: 4}$$

when the lowest vibrational level is taken as energy origin. The product is to be taken over all modes of vibration except that which has to be regarded as stiff. The contribution of the vibrational partition functions to the ratio k_T/k_H is

$$\prod_i \frac{1 - e^{-h\nu_{iH}/kT}}{1 - e^{-h\nu_{iT}/kT}}$$

We shall consider the two factors arising from the rotational and vibrational partition functions as treated above. In his treatment of the same general problem, BIGELEISEN makes use of the fact that certain relationships for isotopic molecules make it possible to eliminate the ratio of the products of the moments of inertia and calculate the partition function ratio from a knowledge of the frequencies only.[1] This possibility follows from a deduction in a paper on isotopic exchange equilibria by BIGELEISEN and MAYER (55) or from the so called product theorem of TELLER and REDLICH (56). In the present case it seems more convenient to discuss the moments of inertia and the vibrational factors as they occur. One reason is that in the treatment by BIGELEISEN the product of all frequencies has to be known rather accurately. Use of the product theorem for the calculation of this product involves a return to the primary expression used here. Another reason is that it is not difficult in the present case to get an idea of the upper limit of the influence of the moments of inertia.

A_T, B_T and C_T are the principal moments of inertia of the activated complex with a tritium atom at the centre of reaction and five protium atoms in the other hydrogen positions. A_H, B_H and C_H are the principal moments of inertia of the activated complex with a protium atom at the reaction centre and four protium atoms and one tritium atom in the other positions. The different possibilities for the position of the tritium will not be treated apart.

In order to give a rough idea of the influence on the moments of inertia of an atom with the mass 3 being placed at a somewhat larger distance from the molecular

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[1] Another difference between the present treatment and that of Bigeleisen is that he uses the form of the specific rate expression, which contains the complete partition function of the activated complex, the effective mass of the activated complex along the coordinate of decomposition, and the length of the potential barrier. The last-mentioned magnitude cancels in the rate ratio, but the ratio of the reduced masses along the coordinate of decomposition has to be inserted. In the present case the factor of the translational partition function, which belongs to movement along the coordinate of decomposition, is removed from the partition function of the activated complex, and the corresponding reduced mass then cancels.

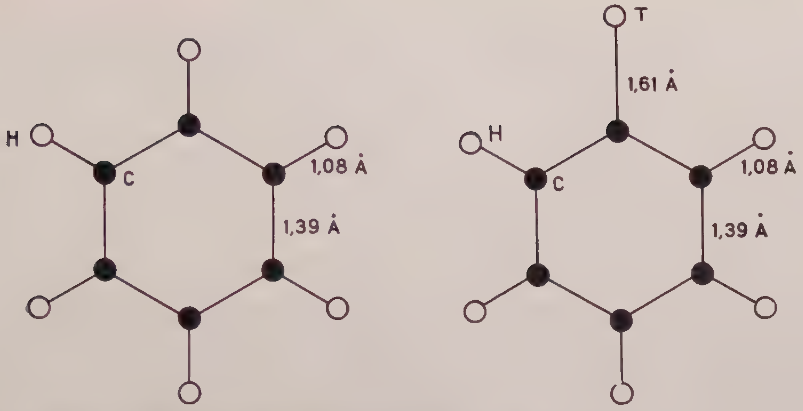

Fig. 7: 1. Figure to the calculation of the moments of inertia of ordinary benzene and a related configuration containing tritium.

centre than usual, the principal moments for an ordinary benzene molecule and those for one with a tritium atom 0.53 Å more distant from the carbon than usual (3.00 Å from the ring centre) have been calculated. The ordinary carbon-carbon distance has been taken as 1.39 Å and the carbon-hydrogen distance as 1.08 Å (Fig. 7: 1).

The ordinary benzene molecule is a symmetrical top. The moment of inertia with respect to an axis in the ring plane is 87.8 (mass-units) · Å^2 and that in respect to the axis perpendicular to that plane twice this value (because the molecule is flat). The product ABC for benzene is accordingly 2 $(87.8)^3$.

The hypothetical tritiumbenzene with a carbon-tritium distance equal to 1.61 Å has two planes of symmetry at right angles and intersecting along a line through the tritium atom and the ring centre. This line is one of the principal axis, and the others are contained in the two planes at right angles to the first one, passing through the centre of gravity. The moment of inertia about the axis through the tritium atom is the same as in the ordinary benzene molecule, or 87.8 (mass-units) · Å^2. The moment of inertia about the other axis in the ring plane is most simply calculated first for an axis through the ring centre parallel to the principal axis and then corrected to the latter. The first moment is found to be 108.7 (mass-units) · Å^2, and the correction is as small as 0.5. Hence the product ABC is $87.8 \cdot 108.2 \cdot (87.8 + 108.2)$. The square root of the ratio between this product and the corresponding one for ordinary benzene is 1.17.

The real activated complex has the entering group in a peripherical position, which tends to increase the moment of inertia and accordingly decrease the influence of the tritium, which will hardly be more peripherical than in the last calculations. Both activated complexes to be compared contain tritium, although in different positions and in different distances from the ring centre. From these facts it may be concluded that the deviation of the factor

$$\left(\frac{A_T B_T C_T}{A_H B_H C_H}\right)^{\frac{1}{2}}$$

from unity is certainly less than that of the value calculated above, 1.17.

If all bonds are very weak and the frequencies low, the vibrational partition functions approximately equal the classical value $\frac{kT}{h\nu}$. The zero-point energies disappear at the same time, and the frequencies occur in the $\frac{k_T}{k_H}$ ratio only as the product $\prod_i \frac{\nu_{iH}}{\nu_{iT}}$. By means of the Teller-Redlich theorem (56) this product may be replaced by the factor $\left(\frac{A_H\,B_H\,C_H}{A_T\,B_T\,C_T}\right)^{\frac{1}{2}}$ in the present case, where the complexes to be compared consist of the same set of atoms. The new factor containing the moments of inertia and that introduced with the rotational partition functions will cancel. Thus no difference in reaction rate would arise if it were possible to treat all vibrations classically, just as no isotopic separation in ordinary exchange equilibria arises in the same case (cf. BIGELEISEN, MEYER (55), who present a more straight-forward deduction of the same thing in connexion with a discussion of exchange equilibria).

If, on the other hand, all atoms are very tightly bound, the frequencies will be high, and the factors $(1 - e^{-h\nu/kT})$ all approach unity. That is the same as saying that all oscillators are in their ground state. The rotational partition functions, which introduce the moments of inertia, and the factor containing the zero-point energies will determine the value of $\frac{k_T}{k_H}$.

The vibrational frequencies in both the ground state and the first excited electronic state of benzene and several of its deuterium derivatives have been investigated by INGOLD et al. (51). A very clear survey has been given by INGOLD in a lecture (52). As has been pointed out earlier it is only the ground state, which is dealt with here.

For the ground state all fundamental vibrational frequencies have been worked out for ordinary benzene, benzene-d, benzene-1,4-d$_2$, benzene-1,3,5-d$_3$, benzene-1,2,4,5-d$_4$, and benzene-d$_6$. Especially ordinary benzene and benzene-d are of interest here.

In the Table 7: I all frequencies below 1000 cm^{-1} in either molecule are given together with the corresponding ratio $\frac{1 - e^{-h\nu_{C_6H_6}/kT}}{1 - e^{-h\nu_{C_6H_5D}/kT}}$ in the cases where its value is highest. Above 1 000 cm^{-1} the value $\nu_{C_6H_6}/\nu_{C_6H_5T}$ is nearly immaterial and even if $\nu_{C_6H_6}/\nu_{C_6H_5T} = \sqrt{3}$ it is below 1.02. It is clear that the product of all vibrational partition function ratios will not be higher than about $(1.02)^{10} = 1.25$. (Only in a few cases $\nu_{C_6H_6} < \nu_{C_6H_5D}$, and in these cases the two frequencies are not very different.)

Thus it may be stated that benzene belongs to the tightly bound molecules.

In the activated complex the partial bonds are supposed to be very weak. All other bonds in the phenyl residue may be supposed to be of the same character as in benzene. In a simplified picture the vibrations of different bonds may be treated as roughly independent. Accordingly we shall discuss those of the partial bonds and those of the phenyl residue separately.

In the vibrational partition function ratio, ν_{iH} refers to a frequency of an activated complex with a protium atom being substituted and a tritium atom in an ordinary carbon-hydrogen bond in the phenyl residue. The ratio factors $\frac{1 - e^{-h\nu_{iH}/kT}}{1 - e^{-h\nu_{iT}/kT}}$ con-

Table 7: I

Certain low vibrational frequencies of benzene in its ground state. (The values of the last column refer to 350° K.)

C_6H_6 cm^{-1}	C_6H_5D cm^{-1}	$\frac{1-e^{-h\nu_{C_6H_6}/kT}}{1-e^{-h\nu_{C_6H_5D}/kT}}$
991.6	980	
605.6	601.8	
(doubly degenerated)		
848.9	849.9	< 1
1110	858	1.02
671	608	1.02
703	698	
985	922	1.005
405	380	1.03
970	995	< 1
848.9	778	1.01

taining vibrational frequencies within the phenyl residue may accordingly be supposed to contribute a factor slightly less than unity, for in most cases $\nu_{iH} \leq \nu_{iT}$. Hence these vibrations are treated as nearly "tight," and the deviations, which occur, tend to decrease the ratio $\frac{k_T}{k_H}$ and thus stress the isotopic effect.

In the case of the hydrogen, which is going to be replaced, nothing is known about the frequencies, but they may be supposed to be low. It is easy to show that at a finite temperature

$$\lim_{\substack{\nu_H \to 0 \\ \nu_T \to 0}} \frac{1 - e^{-h\nu_H/kT}}{1 - e^{-h\nu_T/kT}} = \frac{\nu_H}{\nu_T} \tag{7:5}$$

In the case of this hydrogen atom ν_H is the frequency of the protium and ν_T that of the tritium atom. Hence $\nu_H > \nu_T$, and, as will be shown in the following, the upper limit for the ratio ν_H/ν_T is $\sqrt{3}$. For very low frequencies the vibrational partition function ratio acquires a value close to $\sqrt{3}$. If, as in the preceding, the residue of the complex is considered to be heavy and rigid, it is possible to ascribe three vibrational degrees of freedom to the hydrogen atom in question. However, one of these will correspond to the coordinate of decomposition, and should be excluded in the partition function. Accordingly, the maximum factor, which is introduced with these ratio factors, will be $(\sqrt{3})^2 = 3$. In this simplified picture all vibrations other than the ones treated here are considered to be the same in both complexes, and totally the vibrational partition functions will introduce a factor less than 3 in the expression for k_T/k_H. Of course this treatment involves extensive simplifications, but the result may be supposed not to be very wrong.

As a whole, the ratio of the partition functions of the activated complexes, introduces a factor probably less than 3, even if certain vibrational frequencies are very low.

7 c. The zero-point energy factor. Next the factor

$$e^{-\frac{E_{0T}-E_{0H}}{RT}}$$

is to be discussed. The energy quantities E_{0H} and E_{0T} are the differences between the lowest vibrational level of the activated complex and that of the initial material in the two reactions, the rates of which are to be compared. Therefore it is possible to write in either case:

$$E_0 = E_c + N\left(\Sigma \tfrac{1}{2} h\nu_{\ddagger} - \Sigma \tfrac{1}{2} h\nu_{\text{init}}\right) \qquad (7{:}\ 6)$$

where E_c is the classical activation energy, which corresponds to the difference in energy height on the potential-energy surface. The two sums are the zero-point energies of the activated and initial states respectively, and in order to get molar quantities the sums are multiplied with Avogadro's number N. Since in our present case the initial state is common to both reactions, the difference, which occurs in the exponent is the difference between the zero-point energies of the two activated complexes (ω denotes wave number):

$$E_{0T} - E_{0H} = N\left(\Sigma \tfrac{1}{2} h\nu_{\ddagger T} - \Sigma \tfrac{1}{2} h\nu_{\ddagger H}\right) = \tfrac{1}{2} N h c\left(\Sigma \omega_{\ddagger T} - \Sigma \omega_{\ddagger H}\right) \qquad (7{:}\ 7)$$

As pointed out in the beginning of this chapter the weak point in this reasoning is the lack of knowledge of the hypothetical activated complex. This lack is considerable especially when it comes to a discussion of the influence of the zero-point energy. In the present case many of the vibrational frequencies of benzene will be retained almost unchanged in the activated complex, irrespective of whether protium or tritium is going to be substituted. If we still use a simplified picture with more or less local vibrations, it might be said that the uncertainty concerns the bonds to be created and broken in the reaction, and which are no normal bonds in the transition state.

In the present transition state it is particularly the partial carbon-hydrogen bond, which is of interest. In the initial state this was a normal aromatic carbon-hydrogen bond, and it is certainly considerably weakened on passing into the transition state, where the entering group has also entered the sp^2 orbital. A limit value will be obtained if the frequency and zero-point energy of the weak carbon-hydrogen bond is equalled to 0. The difference in zero-point energy will then only depend on the vibrations in the phenyl residues in the two cases. E_{0T} will correspond to one containing only protium atoms and E_{0H} to one with one tritium and four protium atoms. It seems natural to use the same difference as exists between the corresponding complete molecules, C_6H_6 and C_6H_5T:

$$E_{0T} - E_{0H} = \tfrac{1}{2} N h c\left(\Sigma \omega_{C_6H_6} - \Sigma \omega_{C_6H_5T}\right) \qquad (7{:}\ 8)$$

The difference in the case of tritium is not known, but the corresponding difference between benzene and deuteriumbenzene is known. There is one method to calculate the difference for tritium and it is possible to test the method in the case of deuterium.

It is possible to ascribe the following wave numbers to the vibrational frequencies of a single aromatic C–H bond (INGOLD)[1]:

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[1] The possibility of treating the vibrations of different bonds independently, is a necessary condition for the assumption (made in the beginning of this chapter) that k_H is independent of the position relative to the tritium.

stretching	3000 cm^{-1}
bending in-plane	1100 cm^{-1}
bending out-of-plane	700 cm^{-1}

The zero-point energy is accordingly, in wave-number units, $\frac{1}{2}(3000+1100+700)=$ $=2400\ cm^{-1}$. The oscillator frequencies may be obtained from the formula

$$\omega = \frac{\nu}{c} = \frac{1}{2\pi c}\sqrt{\frac{f}{\mu}} \tag{7: 9}$$

where either f is a force constant and μ the reduced mass of the vibrating system, or f a moment constant and μ the corresponding moment of inertia. In the present case the hydrogen may be looked upon as being attached to a heavy residue. Then the reduced mass will be equal to that of the hydrogen atom, and the moment of inertia proportional to it, since the C–H distance is unchanged in isotopic substitution. The force or moment constants are also unchanged. Hence approximately

$$\omega_D : \omega_H = 1 : \sqrt{2}, \quad \omega_T : \omega_H = 1 : \sqrt{3}$$

and in wave number units (with the observation that E_{0T} refers to a tritium-free aromatic nucleus and Σ' to a single C–H bond only):

$$E_{0T} - E_{0H} = \left(1 - \frac{1}{\sqrt{3}}\right)\frac{1}{2}\Sigma'\,\omega_{C_6H_6} = \left(1 - \frac{1}{\sqrt{3}}\right)\cdot 2400 = 1020\ cm^{-1}$$

or in common energy units

$$E_{0T} - E_{0H} = \tfrac{1}{2}\,N h c\left(1 - \frac{1}{\sqrt{3}}\right)\Sigma'\,\omega_{C_6H_6} = 2900\ \text{cal/mol.}$$

The validity of calculations of this kind may be tested in the case of some of the deuterium-substituted benzenes, the zero-point energies of which are found from their fundamental frequencies.

Table 7: II

Zero-point energies of benzene and some of its deuterium derivatives.

Molecule	$\frac{1}{2}\Sigma\omega$ cm^{-1}	Difference cm^{-1}
C_6H_6	21,536	698
C_6H_6-d	20,838	666
C_6H_6-1, 4-d_2	20,172	666
C_6H_6-1, 3, 5-d_3	19,506	705
C_6H_6-1, 2, 4, 5-d_4	18,801	2×685
C_6H_6-d_6	17,431	

Table 7: II gives these zero-point energies, which are calculated from the results of Ingold et al., and the differences. It is seen that the zero-point energy decreases with an almost constant amount per atom protium replaced by deuterium. (This

principle of linear interpolation has been discussed in one of the papers by INGOLD et al. (54).) According to the present simple method the constant decrement should be

$$\left(1-\frac{1}{\sqrt{2}}\right)\frac{1}{2}\Sigma'\,\omega_{C_6H_6} = \left(1-\frac{1}{\sqrt{2}}\right)\cdot 2400 = 703\ \text{cm}^{-1}$$

which value is in fairly good agreement with those of Table 7: II.

Referring to the result of the last test of the method of calculation, it seems certain, that the difference between the zero-point energies of benzene and benzene-t does not deviate appreciably from the calculated value, 2900 cal/mol. However, the magnitude $(E_{0T}-E_{0H})$ should also include the difference between the zero-point energy components due to the vibrations of the partial bond. The only way to arrive at a definite value in the present calculation is to neglect this difference. It will be particularly high when a hydrogen acceptor is present in the transition state, but fortunately experimental evidence from similar reactions of deuterium exists and shows that the error is not serious.

The lower limit at 350° K for the factor involving the zero-point energies is according to the preceding

$$e^{-\frac{E_{0T}-E_{0H}}{RT}} = e^{-\frac{2900}{2\cdot 350}} = 0.016$$

This factor is by far the most important one in the expression for k_T/k_H.

7 d. The activity coefficient ratio. The last ratio factor to be discussed is the ratio of the activity coefficients of the activated complex. As was mentioned previously the activity coefficients refer to a gaseous standard state in order to make the calculation rest on ideal conditions, under which it is possible to consider all molecular species as being far apart and to use simple expressions for their energy levels. By means of the activity coefficients the equilibrium is transferred to the non-ideal conditions in the solution, and the following equation holds for each species taking part in the equilibrium:

$$c_{\text{gas}} = \alpha\, c_{\text{sol.}} \qquad (7{:}\ 10)$$

The activity coefficient is the ratio between the concentration in the gas phase and that in the solution at phase equilibrium. It may also be looked upon as a correction to the partition function for unit volume of the dissolved substance:

$$F_{\text{gas}} = \alpha\, F_{\text{sol.}} \qquad (7{:}\ 11)$$

When the substance is transferred from the gas phase to the solution, the energy levels are changed owing to the perturbing influence of the environment in the solution.

α is connected to the change in chemical potential caused by the external factors. For the (ideal) gas phase we have

$$\mu_{\text{gas}} = \mu^\circ + RT\ln c_{\text{gas}} \qquad (7{:}\ 12)$$

where μ, is the symbol of chemical potential, and μ° is its value in the standard gaseous state. According to the definition of the activity coefficient, it is possible to write for the solute:

$$\mu_{\text{sol.}} = \mu^{\circ} + RT \ln c_{\text{sol.}} + RT \ln a \qquad (7:13)$$

If the concentration units are the same in the two phases, the two first terms to the right will have the same values for equal concentrations and might be ascribed to internal factors only. The term $RT \ln \alpha$ represents the influence of external factors due to the solvent on the chemical potential:

$$RT \ln a = \Delta\mu; \quad a = e^{\Delta\mu/RT} \qquad (7:14)$$

Correspondingly in the expression for the specific rate, which under ideal conditions may be written

$$k = \varkappa \frac{kT}{h} e^{-\Delta F_{\ddagger}/RT} \qquad (7:15)$$

(cf. equation (6 : 3), $RT \ln K_{\ddagger} = -\Delta F_{\ddagger}$), the activity coefficients introduce a correction for external influences to the standard free energy of activation:

$$\frac{a_A\, a_B}{a_{\ddagger}} = e^{-\Delta F_{\ddagger}\,\text{ext.}/RT} \qquad (7:16)$$

In the present case, where A and B are the same in the two reactions to be compared, only the difference in chemical potential between the two activated complexes has to be considered. It introduces the factor

$$\frac{a_{\ddagger H}}{a_{\ddagger T}} = e^{(\Delta\mu_H - \Delta\mu_T)/RT} \qquad (7:17)$$

Since the complexes are ions $\alpha_{\ddagger}$ will be very small and $\Delta\mu$ a large negative quantity. The reaction, the activated complex of which interacts most strongly with the solvent, will have its α and $\Delta\mu$ most depressed (the latter magnitude in algebraical sense), and be accelerated relatively to the other. Or in other words, the reaction, the activated complex of which is most stabilized by the solvent, is favoured.

The only interaction, which could be supposed to cause a difference in the present case, would be the formation of "hydrogen bonds" with the environment. The only atom to be taken into consideration is that held by the partial bond. Generally hydrogens bound to carbon are not apt to form such bonds, but the hydrogen in question has an acidic character (in next moment it is liberated as a proton or triton) and the complex is positively charged. A comparison of the heats of sublimation of heavy and ordinary water at the triple points gives the difference in internal energy in the case of a strong effect. The heat of sublimation of heavy water exceeds that of ordinary water with 460 cal/mol (57). Although tritium will give a higher difference than deuterium the difference in the effect on the chemical potentials of the activated complexes, which has to be taken into account in the present case, is probably considerably smaller, and since the quantity RT is about 700 cal/mol under the experimental conditions, the relative accelerating influence on the tritium substitution should be rather small.

7 e. Summary of the discussion of k_T/k_H. From the discussion of the different factors, which make up the ratio k_T/k_H the following facts are evident. Owing to the lack of knowledge of the energy surface some rather important data about the activated state are unknown. This is most serious in the case of the zero-point energy of the partial carbon-hydrogen bond, for this energy may be an important counterbalance to that of the initial state, especially if the hydrogen is already bound to an acceptor in the activated state.

The zero-point energy factor is in any case the most important one. If the zero-point energy of the partial carbon-hydrogen bond is neglected, the factor was estimated at 0.016, and the other factors could be supposed to give a factor not higher than 3. Then the ratio k_T/k_H should have the value 0.05.

From the preceding calculations it is obvious that very strong effects are active. In the experimental part results are presented, indicating an equal velocity for protium and tritium nitration within a few per cent under rather varying conditions. This could hardly be a mere coincidence, caused by mutual compensation of the different effects operative in the present hypothetical mechanism.

The result of the discussion might be compared with the results of WYNNE-JONES (33) on the rate of the reaction

$$CH_3 \cdot CH_2 \cdot NO_2 + OH^- \rightarrow CH_3 \cdot \overline{C}H \cdot NO_2 + H_2O$$

for different methylene hydrogen isotopes. He found that under identical conditions a deuteron reacted with about a tenth of the rate of a proton. If the zero-point energy of the methylene hydrogen is assumed to be of the same magnitude as that of an aromatic hydrogen (certainly it is not higher), the difference E_{0T}—E_{0H} would be $\left(1 - \frac{1}{\sqrt{2}}\right) \cdot 2400 = 703 \text{ cm}^{-1} = 2000$ cal/mol. At the temperature of the experiment, 0° C, the zero-point energy factor of the specific rate ratio becomes

$$e^{-\frac{2000}{2 \cdot 273}} = 0.026$$

If in the present case the other factors are supposed to contribute a factor 2, the rate ratio would become 0.05, one half of the experimental value. It is to be observed that the activated complex contains an acceptor for the hydrogen cation, which tends to decrease the difference.

7f. The ratio k'_H/k_H. Another important specific rate ratio is that between protium substitution in a molecule containing only protiums and the same reaction in another containing one tritium (not taking part in the reaction). For the sake of simplicity the ratio shall be discussed for benzene molecules, and it might be supposed that it is possible to transfer the results to other aromatic molecules only with due attention to symmetry differences. Only details being different from those in the preceding discussion will be treated exhaustively.

In both reactions to be compared, a protium is substituted. Thus both are given the index *H*, and the reaction of the hexaprotiumbenzene, which has not occurred before, is denoted by an apostrophe. Benzene is denoted by *A*. The ratio is

$$\frac{k'_H}{k_H} = \frac{\varkappa'_H}{\varkappa_H} \cdot \frac{F'_{\mp H}}{F_{\mp H}} \cdot \frac{F_A}{F'_A} e^{-\frac{E'_{0H} - E_{0H}}{RT}} \cdot \frac{\alpha_{\mp H}}{\alpha'_{\mp H}} \cdot \frac{\alpha'_A}{\alpha_A}$$

The transmission coefficient ratio is certainly very close to unity.

The translational factors of all four partition functions introduce the corresponding molecular weights, in the case of nitration resulting in the ratio

$$\frac{124}{126} \cdot \frac{80}{78} = 1.01$$

The absolute difference of the moments of inertia is very nearly the same for the activated complexes and the initial molecules. One difference, however, counterbalances the other. In paragraph 7 b the principal moments of inertia of ordinary benzene were given. If the same magnitudes for ordinary benzene-t (i.e. with a carbon-tritium distance equal to 1.08 Å) are calculated, the values 87.8, 100.0 and 187.8 (mass-units) . Å^2 are obtained. This gives

$$\left(\frac{A_A\, B_A\, C_A}{A'_A\, B'_A\, C'_A}\right)^{\frac{1}{2}} = 1.10$$

The relative influence of the triton is less in the activated complex and operates in the other direction. Hence all moments of inertia contribute a factor between 1 and 1.10 to the rate ratio.

The symmetry number of both activated complexes is 1. For benzene-t it is 2 and for ordinary benzene 12. The symmetry numbers introduce the factor 6. This means only that there are six positions in benzene equivalent to one specified in benzene-t, specified by the presence of the tritium atom.

It is convenient to compare the rates between one specified position in each molecule, and define the rate constants accordingly. This method shall be followed, and the symmetry factor is then omitted.

The simplified treatment with independent carbon-hydrogen vibrations gives the factor unity for the total contribution from the vibrational partition functions. The centre of reaction is the same in both reactions, and the vibrational factors for the partial bonds cancel. The difference due to the occurrence of a tritium atom, ordinarily bound to carbon, cancels between the ratios $F'_{\ddagger H}/F_{\ddagger H}$ and F_A/F'_A, and in any case the factors for high frequencies are close to unity and very little sensitive to differences in frequency. The zero-point energy factor is also equal to unity. The only approximation involved in this assignment is that the difference between the lowest vibrational energy level of the activated complex and that of the initial state has been considered independent of the presence and position of the tritium atom (common to both states). Contrary to the preceding case, it is not necessary to know the frequencies of the partial bonds in the activated state because they are common to both reactions compared.

Finally the activity coefficients are to be considered. The common aromatic hydrogen is probably not able to form hydrogen bonds, and a difference in interaction with the surrounding medium between protium and tritium must be extremely small. In addition such a difference would cancel largely between $\alpha_{\ddagger H}/\alpha'_{\ddagger H}$ and α'_A/α_A.

It may be concluded that the ratio k'_H/k_H has a value close to unity. The different moments of inertia introduce the largest deviation, which, however, is less than 10 per cent. In the present picture of the mechanism it is accurate enough to assign one and the same specific rate to the substitution of protium, irrespective of the presence of tritium in the molecule. It is then only necessary to use two rate constants, k_H and k_T, for the substitution of protium and tritium, respectively.

8. Application of the theory of absolute reaction rates to the two-step mechanism

8 a. Relation of the mechanism to other theoretical considerations. WHELAND (18) and DEWAR (20) calculate the energies of polar limiting structures, in which a pair of the π-electrons is localized at one carbon atom, and the others form a resonance structure of the same kind as in the pentadienate cation. It is found that the structure of lowest energy has its lone electron pair in the position in which experiments show the substitution to take place most rapidly.

RI and EYRING (21) use the charge distribution of the unperturbed molecule, as it appears in the dipole moment. They find that the attack takes place most easily at the point of highest electron density, and moreover that the relative probabilities of attack in different positions may be calculated from the differences in electrostatic potential at a distance of 1.6 Å from the carbon atom in question, still for the unperturbed molecule.

The unperturbed molecule has a structure intermediate between those for which WHELAND has calculated the energy, and its energy is lower than that of any of them. However, the structure corresponding to energy minimum will be closest to the most stable limiting one. Thus it is not a mere coincidence that the two investigations referred to are in agreement with each other and with experimental facts.

The success of RI and EYRING in predicting accurate values for the relative probabilities of substitution in different positions is a strong support for their picture of the activated complex, though the presence of a positive charge at a distance of 1.6 Å would probably exert a not negligible polarizing influence. (Cf. also the severe criticism by DEWAR (20).) Equal polarizabilities would maintain the charge difference, even if the absolute charges deviate from those of the unperturbed structure. The polarizability of a substituted benzene molecule, however, is certainly not the same in all directions in the ring plane, and generally polarizability decreases with increasing polarization, so the charge difference cannot be supposed to remain unchanged for extensive polarization. The most probable picture of the activated complex seems to fall not very far from that of RI and EYRING with an unperturbed molecule, somewhat modified by the polarizing influence of the attacking cation.

The calculations just discussed involve no energy correction for the loosening of the original carbon-hydrogen bond. Hydrogen is most firmly bound in the positions of highest electron density, and so one and the same lengthening of the bond would require the highest energy in the positions where substitution is most probable. If the loosening of the hydrogen were taken into account in the activated complex of RI and EYRING, smaller energy differences between different positions would result.

The arguments given tend to make the addition mechanism the most probable one. It is possible to imagine an addition product with an almost tetrahedral configuration at the centre of reaction. This requires only a slight deformation of the ring. Now the stretching force constant of a carbon-hydrogen bond is very nearly the same for aliphatic and aromatic molecules, and it might be supposed that a transition from a sp^2 trigonal to a sp^3 tetrahedral configuration would not change the zero-point energy of the bond considerably. We are here particularly interested in the zero-point energies of the activated state of the addition, and the same may be supposed to hold for it, perhaps to a higher degree.

In the very simple case of methane the zero-point energy of a single aliphatic

Table 8: I

Vibrational frequencies of methane.

Frequency, cm^{-1}	Degeneracy
ω_1 2915	1
ω_2 1520	2
ω_3 3022	3
ω_4 1304	3

carbon-hydrogen bond may be calculated as one fourth of the total zero-point energy of the molecule. Table 8: I gives the four different vibrational frequencies of methane and their degeneracy (58).

The zero-point energy of the molecule is found to be 9466 cm^{-1}, and one fourth is 2367 cm^{-1}, or approximately the same value as found for the aromatic carbon-hydrogen bond, 2400 cm^{-1}. The value of the comparison might be discussed, but in any case the result does not contradict the present assumptions.

If a difference exists in the firmness of the carbon-hydrogen bonds, that of the addition intermediate is probably weakest. If so, the equilibrium constant K_{12} of equation (4 : 4) will have a higher value for protium than for tritium, which tends to reinforce the rate difference if the second step is rate-determining.

8 b. The specific rate ratios of the different steps. In the following the different factors of the absolute rate expression for the addition mechanism will be discussed with regard to the influence of the hydrogen mass. The different formulae were found in the preceding paragraphs and will not be given again. The same symbolism will be followed.

The first ratio occurring in k_T/k_H that might be supposed to contribute a factor deviating from unity is the rotational partition function ratio for the two activated complexes, only differing in the position of the triton. In one it is bound by an ordinary aromatic carbon-hydrogen linkage, in the other by a bond tending towards the aliphatic type. The distance from the centre of gravity, however, is nearly the same, and the moments of inertia will not be much different.

Since the rigidity of the two types of carbon-hydrogen bond is about the same, it might be supposed that the complete sets of normal vibrations for the two complexes will give a vibrational partition function ratio not far from unity, especially as its sensitivity to differences in frequency is fairly low for frequencies as high as those coming into question.

The most important factor, the one containing the zero-point energies, may also be supposed to equal unity with good approximation, owing to the same similarity in frequencies.

The ratios of the transmission and activity coefficients cannot deviate much from unity, and accordingly the same may be said about the total ratio k_T/k_H.

As to the ratio k_H'/k_H, the same estimations as were made in the case of the other hypothetical mechanism apply to the present case.

It may be concluded that the realization of the addition mechanism as outlined above would make the three specific rates k_H', k_H, and k_T almost equal and the addition intermediates would be formed with very nearly the same velocities.

The isotopic influence on the second step of the addition mechanism is also of interest since under certain conditions its specific rate appears in the expression for the overall rate.

The second step involves the splitting off of a hydrogen nucleus from a covalent bond of about the same strength as in aliphatic substances. In the case of nitration the intermediate is very similar to the pseudo-acid nitroethane, which was investigated by WYNNE JONES (33) as described in paragraph 7 e. The intermediate should be still more acid and lose the hydrogen cation more easily in consequence of its positive charge. The electron pair joins the four π-electrons, forming the pentadienate cation system of the intermediate. This case is in some respect similar to that of the single-step mechanism, except that no new substituent but a mesomeric system absorbs the electrons. The activated complex contains a weakened bond, and as demonstrated before the zero-point energy difference between a carbon-protium and a carbon-tritium bond gives rise to a considerable difference in the specific rate. The details will not be discussed because the arguments are very nearly the same as in the one-step mechanism. If no base takes part in the activated state, the reaction is of the first order, for which the theory of absolute reaction rates holds as well.

9. Variation of isotopic concentrations in isotopic reactions

9 a. General. The present investigation is based on the competition between hydrogen isotopes for substituting agents. In principle symmetrically situated isotopic atoms within the same molecule compete, but as tritium is used only in tracer amounts, most molecules contain only protium and form the carrier material in all operations. In the measurements the mean tritium content of the total amount of hydrogen is obtained. It is evident that the molecules carrying one tritium atom, among its hydrogen atoms in the reacting position may react with another velocity than those carrying only protium atoms. Consequently the relative concentration of the two kinds will change in the course of the reaction, and the relative amounts having reacted will depend on at what stage the reaction is interrupted. In other words, the measured mean tritium content of the product will be a function of the amount of reaction, if protium and tritium atoms react with different velocities, as they do in the general case.

BIGELEISEN (50) has treated the problem in a very general manner, The following deduction is better suited to the present problem. Equation (9 : 9) may also be obtained as a special case of the final formula of BIGELEISEN by proper substitutions.

It was shown above that it is possible with a fairly high degree of accuracy to use only one rate constant for the substitution of protium irrespective of whether the molecule contains tritium or not. Both the method of preparation and the fact that tritium is used only in carrier amounts prohibit the occurrence of two tritium atoms in the same molecule. The two rate constants for substitution of one specified atom are denoted k_H and k_T.

The aromatic molecule, which is supposed to contain n symmetrical positions for which substitution is possible, is denoted by RH_n. R is assumed to contain no tritium. Three different reactions may occur in the mixture of RH_n and $RH_{n-1}T$ (T denotes tritium):

$$R H_n + X^+ \xrightarrow{n k_H} R H_{n-1} X + H^+$$

$$R H_{n-1} T + X^+ \begin{cases} \xrightarrow{(n-1) k_H} R H_{n-2} T X + H^+ \\ \xrightarrow{k_T} R H_{n-1} X + T^+ \end{cases}$$

where the specific rates are given above the arrows.

If the concentration of RH_n (not radioactive) is denoted by i, that of $RH_{n-1} T$ (radioactive) by a (initial concentrations i_0 and a_0), we find

$$\frac{-da}{-di} = \frac{(n-1) k_H + k_T}{n k_H} \cdot \frac{a}{i} = \frac{n - 1 + \beta}{n} \cdot \frac{a}{i} \tag{9: 1}$$

where β stands for k_T / k_H which may be $\lesseqgtr 1$. If this is integrated:

$$\int_{a_0}^{a} \frac{da}{a} = \frac{\beta + n - 1}{n} \int_{i_0}^{i} \frac{di}{i} \tag{9: 2}$$

$$\frac{a}{a_0} = \left(\frac{i}{i_0}\right)^{\frac{\beta + n - 1}{n}} \tag{9: 3}$$

For the moment the specific activity may be defined as the fraction of a certain molecular species containing tritium. Since the active molecules are very few in comparison with the inactive ones, the fraction is equal to the ratio between active and inactive molecules. Hence

$$\text{Specific activity of initial substance} = \frac{a_0}{i_0} = y_0. \tag{9: 4}$$

From this point it is convenient to discuss the cases $n \geq 2$ and $n = 1$ apart.

9 b. Competition between two or more positions and between molecules. Of the activity of the reacting molecules the fraction

$$\frac{(n-1) k_H}{(n-1) k_H + k_T} = \frac{n-1}{\beta + n - 1} \qquad (> 0 \text{ for } n \geq 2)$$

ends in the derivative, the specific activity of which becomes

$$y = \frac{n-1}{\beta + n - 1} \cdot \frac{a_0 - a}{i_0 - i} \tag{9: 5}$$

The amount of reaction is defined as

$$x = \frac{i_0 - i}{i_0} \tag{9: 6}$$

and (9: 3) is accordingly written

$$\frac{a}{a_0} = (1-x)^{\frac{\beta+n-1}{n}} \tag{9: 7}$$

or

$$\frac{a_0 - a}{a_0} = 1 - (1-x)^{\frac{\beta+n-1}{n}} \tag{9: 8}$$

The equations (9: 4–6) and (9: 8) give:

$$\frac{y}{y_0} = \frac{n-1}{\beta+n-1} \cdot \frac{a_0 - a}{a_0} \cdot \frac{i_0}{i_0 - i} = \frac{n-1}{\beta+n-1} \cdot \frac{1}{x}\left[1 - (1-x)^{\frac{\beta+n-1}{n}}\right] = f(x,\beta) \tag{9:9}$$

Some characteristic features of $f\,(x, \beta)$, regarded as a function of the amount of reaction, are given below. Only positive values of β have real significance and are considered in the following.

$$\lim_{x\to 0} f\,(x, \beta) = \frac{n-1}{n} \quad \text{(independent of } \beta) \tag{9: 10}$$

$$f\,(x, 1) = \frac{n-1}{n} \quad \text{(independent of } x) \tag{9: 11}$$

$$f\,(1, \beta) = \frac{n-1}{\beta+n-1} \tag{9: 12}$$

$$\frac{\partial f}{\partial x} = \frac{n-1}{\beta+n-1} \cdot \frac{1}{x^2}\left[\frac{\beta+n-1}{n}\, x\,(1-x)^{\frac{\beta-1}{n}} + (1-x)^{\frac{\beta+n-1}{n}} - 1\right] \tag{9: 13}$$

$$\lim_{x\to 0} \frac{\partial f}{\partial x} = \frac{(n-1)(1-\beta)}{2\,n^2} \tag{9: 14}$$

$$\left(\frac{\partial f}{\partial x}\right)_{x=1} = \begin{cases} \infty; & \beta < 1 \\ 0; & \beta = 1 \\ -\dfrac{n-1}{\beta+n-1}; & \beta > 1 \end{cases} \tag{9: 15}$$

The function $f\,(x, \beta)$, regarded as a function of the amount of reaction, is pictured in Figs. 9: 1—3 for $n = 2,4$, and 6 and some particular values of β.

The importance of the amount of reaction is quite evident from a study of the function $f\,(x, \beta)$. The curves for different values of β originate from the same point and lie close together for small values of x but spread out rapidly as x approaches unity. The accuracy in the assignment of a certain value to β is a function of the amount of reaction.

If the two isotopes react with the same velocity, $f\,(x, \beta)$ has the constant value $\frac{n-1}{n}$ in the interval $x=0$ to $x=1$. In the experiments on nitration the experimental value y/y_0 equals $\frac{n-1}{n}$ within the experimental error. In most of the ex-

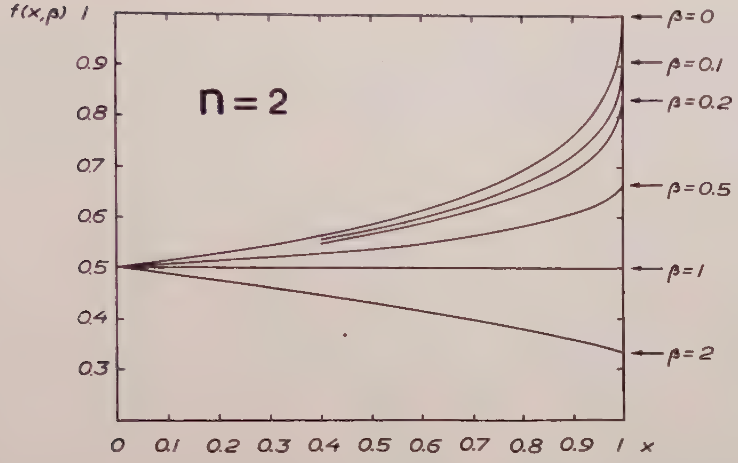

Fig. 9: 1. The fraction of tritium retained in the derivative, $f(x, \beta)$, as a function of the amount of reaction (x) for some particular values of β; competition between two positions.

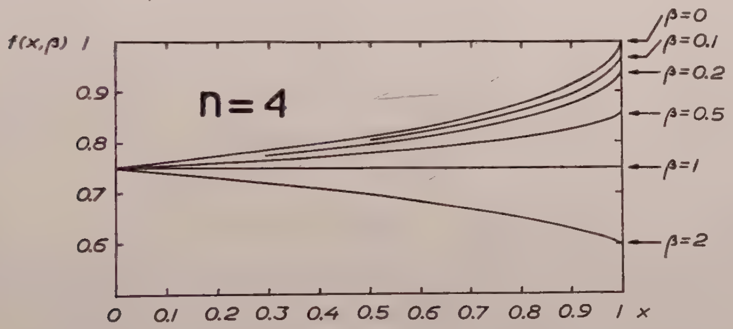

Fig. 9: 2. The fraction of tritium retained in the derivative, $f(x, \beta)$, as a function of the amount of reaction (x) for some particular values of β, competition between four positions.

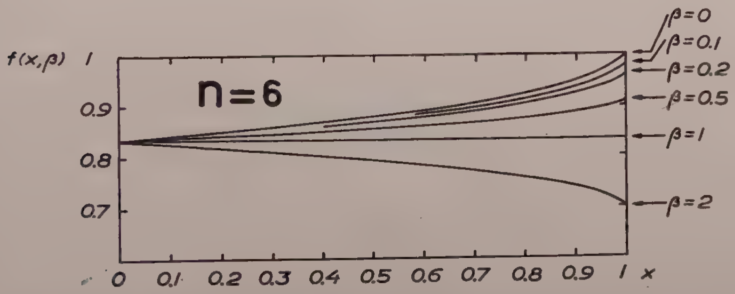

Fig. 9: 3. The fraction of tritium retained in the derivative, $f(x, \beta)$, as a function of the amount of reaction (x) for some particular values of β, competition between six positions.

periments the amount of reaction is not far from unity, and consequently it may be concluded that β does not deviate much from unity. The accuracy with which β equals unity has to be determined from the accuracy in the determination of y/y_0 and the amount of reaction. For that purpose it is necessary to study $f(x, \beta)$ as a function of β, since it is impossible to solve equation (9: 9) for β explicitely. Thus it is found:

$$\frac{\partial f}{\partial \beta} = \frac{n-1}{(\beta+n-1)^2} \cdot \frac{1}{x} \left[(1-x)^{\frac{\beta+n-1}{n}} - \frac{\beta+n-1}{n} (1-x)^{\frac{\beta+n-1}{n}} \ln (1-x) - 1 \right]$$

and

$$\frac{\partial^2 f}{\partial \beta^2} = \frac{n-1}{(\beta+n-1)^3} \cdot \frac{1}{x} \left[2 \frac{\beta+n-1}{n} (1-x)^{\frac{\beta+n-1}{n}} \ln (1-x) - \right.$$
$$\left. - \left(\frac{\beta+n-1}{n} \right)^2 (1-x)^{\frac{\beta+n-1}{n}} \ln^2 (1-x) - 2 (1-x)^{\frac{\beta+n-1}{n}} + 2 \right] \qquad (9: 17)$$

The value $\beta = 1$ is of particular interest, and the corresponding values of the derivatives are:

$$\left(\frac{\partial f}{\partial \beta} \right)_{\beta=1} = - \frac{n-1}{n^2} \cdot \frac{1}{x} [(1-x) \ln (1-x) + x] = - \frac{n-1}{n^2} \cdot \frac{1}{\varphi (x)} \qquad (9: 18)$$

$$\left(\frac{\partial^2 f}{\partial \beta^2} \right)_{\beta=1} = \frac{n-1}{n^3} \cdot \frac{1}{x} [2 (1-x) \ln (1-x) - (1-x)\, ln^2 (1-x) + 2x] \qquad (9: 19)$$

In the interval $x = 0 \rightarrow 1$ $\left(\frac{\partial^2 f}{\partial \beta^2} \right)_{\beta=1}$ increases monotonically from 0 to $\frac{2(n-1)}{n^3}$, which means that the curve for $f(x, \beta)$ as a function of β is concave upwards and only slightly curved in the neighbourhood of $\beta = 1$ for all occurring values of x. Fig. 9: 4 shows $f(x, \beta)$ for some particular values of n and x.

By means of these curves it is possible to get the accuracy in the determination of β from the accuracy in y/y_0. However, since the curves coincide closely with their tangents (also represented in Fig. 9: 4) for a fairly long range, the differentials may be replaced by differences even for considerable deviations from $\beta = 1$. In addition, for $\beta < 1$, which direction is of most interest, the curvature as discussed above makes the real error determined from the curve slightly smaller than that determined approximately from the tangent. From equation (9: 18) follows:

$$\Delta \beta = - \frac{n^2}{n-1} \varphi (x) \Delta f \qquad (9: 20)$$

The relative deviation of the experimental y/y_0 from $f(x, 1) = \frac{n-1}{n}$ is denoted by α. Hence

$$\Delta f = \alpha f(x, 1) = \alpha \frac{n-1}{n} \qquad (9: 21)$$

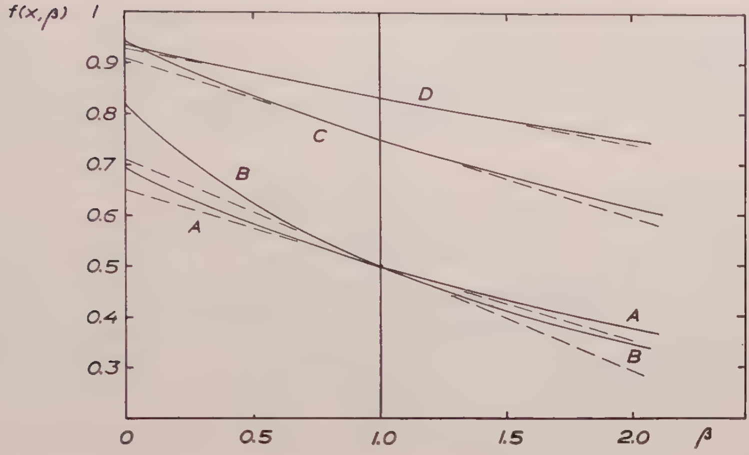

Fig. 9: 4. The fraction of tritium retained in the derivative, $f(x, \beta)$, as a function of β for some particular numbers of competing positions (n) and amounts of reaction (x): A, $n = 2$, $x = 0.8$; B, $n = 2$, $x = 0.95$; C, $n = 4$, $x = 0.95$; D, $n = 6$, $x = 0.85$.

and the corresponding deviation of β from unity is

$$\Delta\beta = -n a \varphi(x) \tag{9: 22}$$

The function $\varphi(x)$ was defined in equation (9: 18), and some values are given in Table 9: I.

Table 9: I

The function $\varphi(x) = \dfrac{x}{(1-x)\ln(1-x)+x}$

x	$\varphi(x)$	x	$\varphi(x)$	x	$\varphi(x)$
		0.75	1.859	0.95	1.187
0.50	3.258	0.80	1.673	0.96	1.155
0.55	2.884	0.85	1.503	0.97	1.122
0.60	2.566	0.90	1.344	0.98	1.087
0.65	2.300	0.92	1.281	0.99	1.049
0.70	2.066	0.94	1.219	1.00	1.000

The error in β is proportional to the number of positions available for substitution and to the relative error in y/y_0 and increases rapidly with decreasing x. The most favourable conditions for experiments with competition within the same molecule is to have only two positions available and to make the conversion as quantitative as possible.

In some of the calculations $f(x, \beta)$ and β occur in the same equation. In order to get an approximate solution of the transcendental equation in the neighbourhood of $\beta = 1$ it is convenient and legitimate to replace $f(x, \beta)$ with its tangent in $\beta = 1$. The corresponding Taylor expansion is:

$$f(x, \beta) = f(x, 1) + (\beta - 1)\left(\frac{\partial f}{\partial \beta}\right)_{\beta=1} =$$

$$= \frac{n-1}{n} + (1-\beta)\frac{n-1}{n^2}\cdot\frac{1}{\varphi(x)} = \frac{n-1}{n}\left(1 + \frac{1-\beta}{n\varphi(x)}\right) \tag{9: 23}$$

in which the equations (9: 11) and (9: 18) have been used.

The function $f(x, \beta) = \frac{y}{y_0}$ gives the relative change in content of active molecules, caused by the reaction. Since all kinds of hydrogen atoms are mixed in the analytical procedure, it is generally not y and y_0 that are measured. If the original molecule contains n hydrogen atoms in the reactive positions, and in addition m hydrogen atoms in other positions, the reaction could be written

$$R'H_{m+n} + X^+ \rightarrow R'H_{m+n-1}X + H^+$$

(The symbol R used before corresponds to $R'H_m$, and it was assumed to contain no tritium.) The magnitude measured is the proportion z of tritium in the total amount of hydrogen, and the corresponding relative change is

$$\frac{z}{z_0} = \frac{y}{y_0}\cdot\frac{m+n}{m+n-1} \tag{9: 24}$$

Since m has no immediate interest in the discussion, $\frac{y}{y_0}$ is calculated at once from the measured ratio $\frac{z}{z_0}$ according to

$$\frac{y}{y_0} = \frac{m+n-1}{m+n}\cdot\frac{z}{z_0} \tag{9: 25}$$

In several cases only the final result of two consecutive reactions is observed. In principle this involves nothing new, and the foregoing treatment may be applied to each step separately, with the exception that the residue R or $R'H_m$ is generally not free from tritium in the second step. This fact requires some modification in the application of $f(x, \beta)$ to that step. The general treatment of this would be too unwieldy and is replaced by a separate treatment in each particular case.

9 c. Competition between only two kinds of molecules. If in the foregoing treatment $n = 1$, $f(x, \beta)$ equals zero, because no tritium is contained in the derivative. The tritium is either lost in the reaction, or if its reaction rate is low, it is prevented from reacting. The simple reaction system in cases like this may be represented by:

$$LH + M \xrightarrow{k_H} X + YH$$

$$LT + M \xrightarrow{k_T} X + YT$$

with the specific rates given above the arrows. It might be of interest to follow the variation in specific activity of the reactant LH and also of the resulting YH. In the substitutions the converted hydrogen is generally lost, but the main interest in YH is connected with the ignition and reduction reactions of the analytical procedure.

Equation (9: 3) with $n = 1$ gives

$$\frac{a}{a_0} = \left(\frac{i}{i_0}\right)^{\beta} \tag{9: 26}$$

The relative change in the specific activity of the reactant is

$$\frac{a}{i} \cdot \frac{i_0}{a_0} = \left(\frac{i}{i_0}\right)^{\beta-1} = (1 - x)^{-(1-\beta)} = g\,(x,\,\beta) \tag{9: 27}$$

The corresponding change between the initial LH and the resulting YH is

$$\frac{a_0 - a}{i_0 - i} \cdot \frac{i_0}{a_0} = \frac{1}{x}[1 - (1 - x)^{\beta}] = h\,(x,\,\beta) \tag{9: 28}$$

The functions $g\,(x,\,\beta)$ and $h\,(x,\,\beta)$ as functions of x are illustrated for different values of β in Figs. 9: 5—6. For the latter function some values are also given in Table 9: II.

Table 9: II

$$h\,(x,\,\beta) = \frac{1}{x}[1 - (1 - x)^{\beta}]$$

β	x	$h\,(x,\,\beta)$
0.5	$1-10^{-5}$	0.997
0.2	$1-10^{-5}$	0.90
	$1-10^{-10}$	0.99
0.1	$1-10^{-5}$	0.68
	$1-10^{-10}$	0.90
	$1-10^{-20}$	0.99

These extreme values cannot be read off from the curve, and they show that the lagging of the slow-reacting isotope is very pronounced even for moderate differences in specific rate. As pointed out in the discussion of the analytical method, it seems surprising that it should be possible to obtain such reproducible results as have actually been obtained.

Fig. 9: 7 shows $g\,(x,\,\beta)$ as a function of β for two particular values of x.

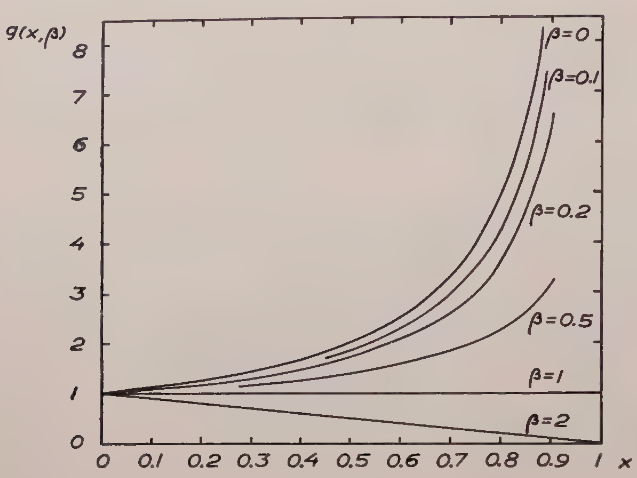

Fig. 9: 5. Competition between two kinds of molecules only. The tritium content of the remaining initial compound, $g(x, \beta)$, as a function of the amount of reaction (x) for some particular values of β.

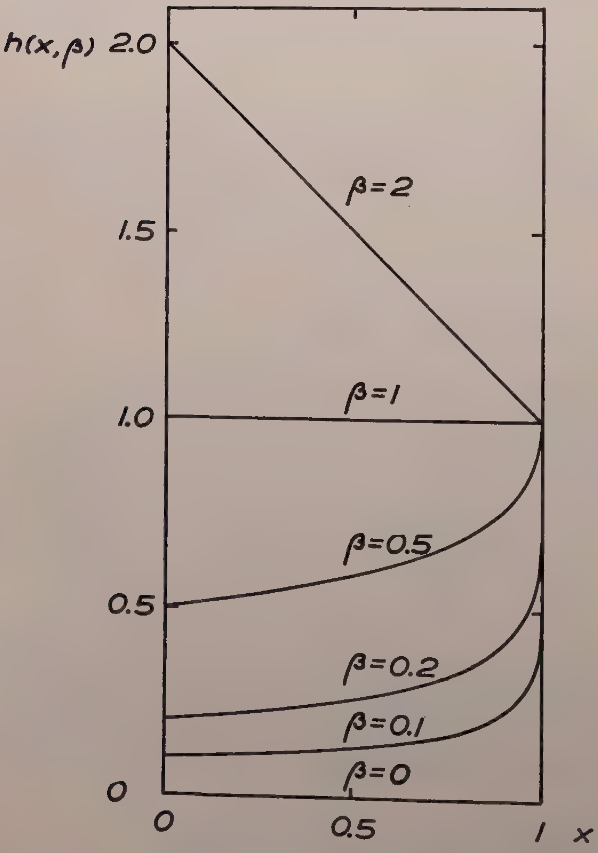

Fig. 9: 6. Competition only between two kinds of molecules. The tritium content of the product, $h(x, \beta)$, as a function of the amount of reaction (x) for some particular values of β.

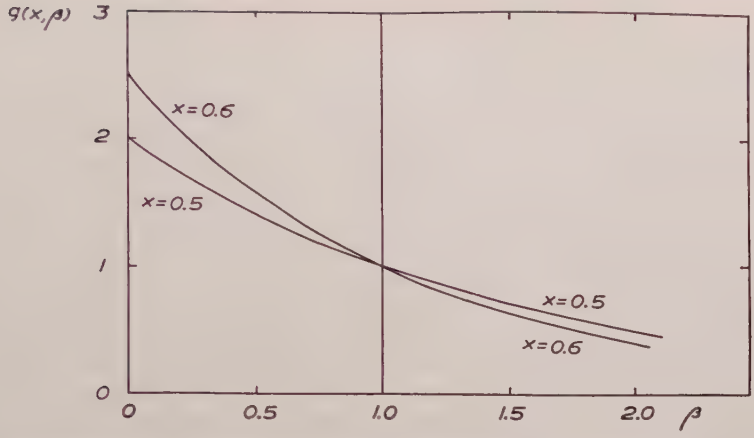

Fig. 9: 7. Competition only between two kinds of molecules. The tritium content of the remaining initial compound, $g(x, \beta)$, as a function of β for two particular values of the amount of reaction (x).

IV. Experimental part

10. The determination of tritium

The determination of tritium in water has been described by the present writer in another paper (59). The same method has been used throughout this investigation, and only a critical discussion of its reliability will be given here. In addition the combustion of the organic compounds will be described shortly and its sources of error discussed.

10 a. The unit of specific activity. During the whole investigation the Geiger-Müller tube was unchanged. Consequently it was possible to use a practical unit for the specific activity, referring to the particular tube. This was taken as that recorded by the mechanical register per minute. The geometrical volume enclosed by the cathode is about 70 cm³. At the standard hydrogen filling pressure of 90 mm, this volume corresponds to about 0.34 millimole. The effective counting volume may be estimated at about that corresponding to a fourth of a millimole. Since a scale of 64 is used, one unit recorded by the register per minute corresponds to about 250 disintegrations per millimole hydrogen per minute.

10 b. The combustion of tritium compounds. Since the best standard method for the determination of tritium requires elementary hydrogen, which is easily obtained by the reduction of water, all compounds were burned and the water collected.

Nitrogen-free substances were burned over red-hot copper oxide in a stream of oxygen. When the compound contained halogen, the combustion gases were led through a second tube containing heated silver wool and a second layer of cupric oxide in order to get pure combustion water.

Substances containing nitrogen were surrounded with powdered cupric oxide and burned in the same manner but in a stream of carbon dioxide. The combustion gases were led through a second tube containing reduced and heated copper, also in order to get pure combustion water. When halogen was present, the tube containing layers of silver wool and cupric oxide was intercalated before that with reduced copper.

The sodium sulphonate, finally, was burned according to the well-known procedure of REIHLEN and WEINBRENNER. The sample was mixed with molten and powdered potassium dichromate in a copper boat, and the combustion layer contained platinized asbestos, silver wool, and "Vinosit B." Streaming oxygen was used.

The combustion gases were passed through a freshly baked receiver, cooled with an ice-sodium chloride mixture. The temperature was —10° C or below. At 0° C the fraction of the water vapour passing through without condensing was shown to be less than 4 %, and according to the vapour pressure the loss should be half that amount or 2 % at —10° C. Since the vapour pressure of HTO is certainly less than that of H_2O, the condensed part will be richer in tritium than the initial vapour. If the relation between the mole fractions in the vapour phase and the condensate is governed by Raoult's law, the ratio between the tritium concentration in the condensed part and that in the initial vapour is $(\alpha+\beta-\alpha\beta)^{-1}$, where α denotes the ratio of the vapour pressures of pure HTO and H_2O and β the fraction condensed. If $\beta = 0.98$, the error is less than 1 % if α has a value between 0.5 and 1, which is certainly the case. A successive condensation in different zones or successive freezing of the condensate may cause the vapour residue to be in equilibrium with a phase poorer in tritium than the average condensate. This would tend to increase the error, as would also be done by incomplete mixing of the condensate from different zones. Such a mixing is difficult with amounts of water as small as 30–40 mg, and the condensate was only centrifuged down to the bottom of the receiver. It could not be avoided, however, that some moisture stuck to the upper part of the walls.

All these sources of error, as well as a probable lagging of the heavy isotope in the combustion (cf. paragraph 9 c) would seem to be able to cause considerable errors in the tritium concentration. Several parallel determinations have been carried out, however, and their results are given in a following paragraph. They show an astonishing agreement, thus proving that the different separation effects do not affect the result to such an extent as could be feared.

The water, which was collected at the bottom of the receiver by means of centrifugation, was immediately enclosed in an ampoule and the tritium content was then determined.

10 c. Critical discussion of the determination of tritium in water. The method has been described in the paper referred to (59).

One could fear that also the reduction of the water by means of magnesium metal should suffer from the same sources of error as the combustion, i.e. considerable lagging of the heavy isotope. The effect would probably be more serious in the present case, because no streaming carrier gas is used in the process. Thus the reduction will always be incomplete owing to the dead space in the end of the furnace tube. The only way to justify this procedure is to point to the empirical facts.

The dilution of the active with inactive hydrogen would involve no appreciable error, because the room temperature did not change during the short period of dilution, and no heat effects of the gas transfer could be observed as slow changes in pressure after the dilution. The gas law must hold very accurately, and the pressure could be read to a few tenths of a per cent.

The temperature at the filling of the Geiger-Müller tube was generally not controlled. The temperature of the room, however, did not change very much, and even deviations as large as ±2° from the mean will cause deviations less than ±0.7 % in the true hydrogen content of the tube at the standard pressure. The pressure itself could, as previously, be controlled within a few tenths of a per cent.

The Geiger-Müller tube worked very evenly and did not change its properties during the course of all the experiments (cf. next paragraph). In all cases the entire plateau was determined, at least five points, and at least 4000 counts were taken for each (generally several times more). The mean error in such a point is consequently $\pm (4000)^{\frac{1}{2}}$ and the relative one $\pm (4000)^{-\frac{1}{2}} = \pm 1.6\,\%$. If the plateau position is considered as the mean of 5 such determinations, the mean error will be ± 0.8 %. At a further increase of the accuracy, irregularities in the operation of the tube become predominant.

Typical results are shown graphically in the paper referred to (59). In its Fig. 3 the radii of the circles representing the experimental points are of about the same size as the mean error (the statistics were slightly worse than in later experiments). The same is the case for Fig. 4, where the relative error has been halved. In Figs. 5 and 6 of the same paper the circles are not at such a simple scale. It is evident from them, however, that the limited resolving power causes no serious error below 6000 counts per minute, and generally no measurements were made above half that counting rate.

10 d. The reproducibility of the determination of tritium in organic compounds. Parallel determinations were carried out on several samples. Table 10: I is a complete list of these and gives the two values (in the common unit of specific activity) and the difference in per cent. If the determinations were carried out at different dates, they have been corrected to the same date by assuming 12.0 years as the half-life of tritium (60–62).

The mean value of the differences is 1.8 %. Of the 18 differences 16 are equal to or less than 3.1 and only one falls outside 4.0 %. In all these determinations the two water samples were prepared in different combustions.

The constancy of the efficiency of the Geiger-Müller tube is evident especially from the determinations on toluene-4-t and naphtalene-α-t. The time interval between the determinations were respectively 523 and 357 days, and the corrections for decay 7.9 and 5.5 %. Practically all determinations fall within the period covered by at least one of these intervals.

In order to control the linearity two samples of toluene-4-t were compared. One was prepared by dilution of the other 13.4 times. The ratio of the specific activities was found to be 1: 13.3.

Some results, which will be given in the following, permit a comparison between determinations on different types of compounds. Thus, for instance, toluene-3-t and toluene-α-t have been converted into their 2,4-dinitro derivatives, and the activities before and after the conversion were compared. If the compounds were not contaminated with other isomeric tritium derivatives, no tritium should be lost in such reactions. The results fall within the same limits of experimental error as those above though isomeric impurities would imply a new source of error. The detailed results will be given in connexion with the nitration of toluene and bromobenzene.

Table 10: I

Parallel determinations of tritium.

Substance	Results (for unit, cf. paragraph 10 a)		Difference, %
Benzene-t	31.2	31.8	1.9
»	47.4	48.7	2.7
»	16.8	17.0	1.2
Toluene-4-t	37.1	37.5	1.1
Toluene-α-t	7.85	8.10	3.1
Bromobenzene-2-t	10.4	10.5	1.0
»	24.5	25.0	2.0
Bromobenzene-3-t	26.5	26.7	0.8
Bromobenzene-4-t	92	92	0
»	58.9	59.0	0.2
Naphthalene-α-t	24.0	24.1	0.4
2,4-Dinitrotoluene-3-t and -5-t	6.60	6.87	4.0
α-Nitronaphthalene-α'-t	21.0	22.3	6.0
p-Dibromobenzene-t	32.4	32.4	0.0
»	5.24	5.31	1.3
»	55.9	57.1	2.1
Bensophenone-t	56.2	57.6	2.5
Sodium benzenesulphonate-t	17.9	18.2	1.7

11. The preparation of tritium compounds

11 a. The preparation of tritium. In the paper on the determination of radioactive hydrogen (59) the preparation of tritium-containing water was described. The same method has been followed throughout this investigation. In most of the experiments water of about ten times higher specific activity than that given in the paper referred to has been used, or about 10^7 disintegrations per millimole per minute.

11 b. General discussion of preparative methods. The starting material for the synthesis of organic tritium compounds has been water, just as is generally the case in the synthesis of deuterium compounds, and the methods used for the latter could also be used for the former. In all cases, except in the preparation of benzene, it has been necessary to introduce the tritium into one definite position. Contrary to most work on deuterium compounds, it is not necessary in the present case to take precautions against moisture and other sources of light hydrogen, because the tritium is always present only in tracer amounts, and the bulk of material is constituted of ordinary hydrogen compounds.

Langseth (63) has given a survey of the different methods available for the preparation of isotopically pure organic deuterium compounds. From this it is evident that not very many methods are available for the present purpose, and some of the classical methods of replacing groups by hydrogen are useless for different reasons.

The decarboxylation of salts of carboxylic acids has been investigated thoroughly by Weldon and Wilson (64), who found that extensive hydrogen randomisation took place between the reactants, even between calcium deuteroxide and benzene, at the temperature of the reaction.

The hydrolysis of sulphonic acids is inconvenient, because the reaction is carried out with aqueous acid, which involves a waste of isotopic hydrogen, and very probably exchange of hydrogen will also take place under the same conditions.

The former objection applies also to the reduction of diazonium salts in its general forms. The most favourable reducing agent in the present case seems to be alcohol, since only the hydrogen of the oxy group is apt to exchange with other protons, e.g. those of some added isotopic water. Such an experiment was carried out in the case of m-nitroaniline in order to prepare nitrobenzene-3-t, but the result showed that this hydrogen probably does not enter the aromatic nucleus. Presumably a methylene hydrogen replaces the diazonium group, and alcohol containing labelled hydrogen in this position cannot be prepared so simply. The experiment will be described in detail in another part of the present paper.

Finally there remains the method of decomposition of Grignard compounds. At least in the case of aromatic compounds this method is by far the best one. It is carried out in ether as a solvent, the hydrogens of which do not interchange with various donors of hydrogen nuclei under the conditions of the experiment, and no case of formation of isotopic compounds isomeric to those corresponding to the original halogen compound is known hitherto. However, it is known that in certain cases Grignard reagents such as benzylmagnesium chloride will give anomalous products (65), and care should be taken in such cases, even if a rearrangement in the simple decomposition does not seem very probable. Nothing in the results obtained in the present investigation seems to indicate a rearrangement, and the small deviations are of such an order of magnitude, that they may very well be explained by the occurrence of traces of isomerides in the organic halides. The difficulties met with in the preparation of benzene-1,4-d_2, which was contaminated with benzene-d, owing to the formation of dimagnesium Grignard complex and its acquiring light hydrogen from the solvent (66), are of importance only for the preparation of isotopically pure substances.

The fact that the Grignard reaction is the only practical reaction for the introduction of tritium into definite positions puts some limits to the choice of compounds. The residue R in the Grignard complex RMgX may be a hydrocarbon and also a halogenated hydrocarbon residue. With nitro groups, however, Grignard reagents react in a complicated manner (67), and so the method cannot be used for the preparation of tritium-substituted nitrobenzenes. That was why the reduction of diazonium compounds was tried for the same purpose, as mentioned above.

In the case of benzene, finally, all hydrogens are equivalent, and, besides the Grignard method, exchange reactions could be used. In the present work both were practised, the latter in the form used by INGOLD, RAISIN, and WILSON (41), i.e. exchange with aqueous sulphuric acid.

11 c. The Grignard reaction in practice. The introduction of isotopic hydrogen into definite positions of aromatic nuclei by the use of Grignard compounds has been thoroughly investigated, especially by LANGSETH and KLIT, and by WELDON and WILSON.

One of the practical difficulties is caused by the hygroscopic nature of the basic magnesium halide formed in the decomposition with water added to the Grignard solution. If the reaction is to be completed in this way, a large excess of isotopic water has to be used. Since the water is the most valuable reactant, the method has to be modified.

LANGSETH and KLIT (68) used deuterium chloride instead of heavy water for the decomposition. Besides the fact that no isotopic hydrogen is lost by absorption in the magnesium salt formed, the method makes the complete utilization of the hydrogen theoretically possible, which is not the case with water. In the latter case only one of the hydrogens of the water enters the aromatic compound, and 50 % is the maximum yield if equal reaction velocities are assumed for the isotopes. The hydrogen chloride, however, is formed by the reaction

$$H_2O + SOCl_2 \rightarrow 2\,HCl + SO_2$$

i.e. without any loss of hydrogen, and all the hydrogen of the chloride ends in the aromatic compound.

By this method it is possible to prepare polydeuterated benzenes from the corresponding halides in one single operation, because the dry hydrogen chloride does not attack magnesium metal. LANGSETH and KLIT prepared all partly deuterated benzenes in this manner with good yields with respect to both the organic halide and the heavy hydrogen. In the case of benzene-d, benzene-1,2-d_2, -1,3-d_2, and -1,4-d_2 the yields in respect to deuterium were 28, 22, 23, and 33 % respectively. According to the authors the method was improved somewhat after these preparations had been made. WELDON and WILSON (66) repeated the preparation of benzene-1,4-d_2 and got 38 % yield with deuterium chloride and 64 % with deuterium bromide. The yield in respect to the organic halide was generally higher, for the heavy water was used in excess. If the yield in respect to deuterium was the same in each step of the preparation of the dideutero compounds, the yields per step obtained by LANGSETH and KLIT were 46, 47, and 57 % respectively and those by WELDON and WILSON 61 and 80 % respectively. The figures were calculated on the assumption of isotopic purity, but the deuterium content of the dideutero benzenes was generally found to be somewhat less than the theoretical one, because some protium was taken from the solvent.

WELDON and WILSON (66) prepared benzene-d by the decomposition of dry Grignard compound with heavy water. In this manner the amount of ether to be separated from the benzene was diminished. They used twice the amount of water equivalent to the Grignard compound and got a yield of 40 %. As this is probably calculated on the basis of the halide, the corresponding yield in respect to deuterium is 10 % (theoretical maximum 50 %).

11 d. The present preparations by means of the Grignard reaction. In the present preparations of tracer amounts of benzene-t, the four different toluene-t isomerides, naphthalene-α-t, and the three bromobenzene-t isomerides still another method was followed. Some of the laborious details of the other methods could be avoided without impairing the yield too much. Advantage could also be taken from the circumstance that it was unnecessary to take isotopic purity into consideration.

The filtered Grignard solution was poured into another portion of dry ether, in which an amount less than equivalent to the Grignard complex of radioactive hydrochloric acid had been dissolved. During this process, the solution was shaken vigorously. Hydrochloric acid instead of pure water was used in order to increase the theoretically possible yield a little and diminish the amount of basic magnesium halide formed. Since the Grignard was poured into the solution containing water, clogging together of magnesium salt, which might easily enclose parts of the solution introduced in the opposite case, was avoided. The addition was carried out rather rapidly in order to diminish the absorption of the moisture in the magnesium salt already formed, and external cooling was necessary, as well as the use of a reflux condenser.

After some minutes, when the isotopic water had reacted, the decomposition was completed with ordinary water and hydrochloric acid. The resulting solution was washed thoroughly with water, a solution of sodium carbonate or better sodium hydroxide, water again, and finally dried with calcium chloride. The ether was distilled off through a Widmer or a glass bead column.

When the product was benzene or toluene, which have rather low boiling points, the distillation was interrupted at about 36° C. The remaining ether was then removed by simple extraction with three portions of 60 % perchloric acid, which is known to cause no hydrogen exchange (69). If the two layers were difficult to separate sharply, the aqueous phase was shaken with some of the corresponding inactive hydrocarbon to take care of the last traces of active compound, and this portion was added to the main part. The organic liquid was washed with water, sodium carbonate solution, water again, and dried with calcium chloride.

The benzene or toluene was finally distilled twice from sodium in a small Podbielniak column at atmospheric pressure. The first time the hydrocarbon was heated gently with the sodium before the beginning of the distillation in order to complete the drying. In the first distillation only the main fraction, boiling within a narrow temperature interval, was collected. In the second one almost all of the liquid conformed to these demands. In most transfers, inactive hydrocarbon was used to wash out the last traces of active compound.

Since the last part of the treatment of benzene and toluene involved contact with metallic sodium and the phosphor bronze spiral of the column at the boiling point temperature, one experiment was made in order to show that no hydrogen exchange occurs as a result of catalytic influences. Thus inactive cumene and toluene-4-t were treated together according to the preceding description from and including the treatment with perchloric acid. Since cumene has a higher boiling point than toluene, the temperature conditions at the contact with the metals will be similar to those of the separation of toluene from a heavier residue. After separation from toluene the cumene showed a very faint (if any) activity. In the case of toluene-4-t appreciable amounts of p,p′-bitolyl were formed as a by-product and its specific activity was shown to be less than 1 % of that of the toluene. Thus no appreciable exchange seems to take place and the activity found in the last case might be supposed to originate from impurities.

If no exchange occurs between different aromatic molecules of similar kind, exchange of hydrogen between different positions within the same molecule seems highly improbable.

In the preparation of the bromobenzenes practically all ether was distilled off on a water bath, and the residual ether evaporated in vacuum. Owing to the large difference in boiling point between the ether and the bromobenzene it is possible to obtain a complete separation without appreciable loss of the latter. The bromobenzene was finally distilled twice at a pressure of some centimeters of mercury and only the constant boiling fractions collected.

In the case of toluene-α-t benzyl chloride was used as the starting material. In all other cases the bromine compounds were used. In one attempt to prepare bromobenzene-2-t, 1-bromo-2-iodobenzene was tried as starting compound. The preparation of bromine compounds from diazonium salts may sometimes lead to a product contaminated by unwanted bromine compounds owing to bromination by free bromine, liberated by the nitrite, and it might be safer to introduce iodine. It was hoped that the magnesium would react preferentially with the iodine as compared to the bromine in the mixed molecule, but no constant boiling fraction corresponding to bromobenzene was obtained.

The ordinary batch was about 0.1 mole of organic halide and magnesium, and the Grignard reaction was carried out in the ordinary way. With the dibromobenzenes all of the halide was in some instances added from the beginning, and the reaction was then easily regulated by means of outside cooling. No significant difference was found between freshly turned magnesium and the commercial turnings in most cases, except with ortho-dibromobenzene, which reacted more readily with the former. Some of the more durable reactions were carried out in a nitrogen atmosphere.

The decomposition was made by means of generally 10 to 20 mg of water containing tritium in 0.5 to 1 ml of concentrated hydrochloric acid, which was all dissolved in 150 to 250 ml of dry ethyl ether.

In the following some particular data are given about the individual preparations, to which the foregoing applies where not otherwise indicated. The "activity yield" is the percentage of the tritium used in the decomposition that ends in the final product. It was determined from the weight and specific activity of the water used and the same magnitudes of the product. Since the small amount of active water is introduced in a much larger amount of concentrated hydrochloric acid, the maximum percentage attainable is about 56 % if the same quantitative behaviour might be ascribed to the two isotopes in the decomposition reaction. Sometimes the side reactions of the Grignard reagent were more extensive than expected, and therefore the hydrochloric acid might have been in excess in practice though not in theory. This is the probable explanation of at least some of the low activity yields. Generally portions of the inactive compound were used for the washing of vessels and extraction liquids and similar purposes, and consequently no yield calculated on the halide can be given. All temperatures given are uncorrected.

Benzene-t I. Commercial bromobenzene (Merck & Co., U. S. A.) was used for the Grignard reagent. The final product boiled at 78.9–79.6° C. Activity yield 21 %.

Benzene-t II. The same Grignard reagent as for benzene-t I was used. B. p. 78.5–79.8° C, $n_D^{20.2} = 1.5008$. Activity yield 25 %.

Toluene-2-t I. o-Bromotoluene (m. p. —27––25° C, b. p. 177–180° C, $n_D^{20.9} = 1.5556$) was prepared from o-toluidine (Merck, Darmstadt) according to Organic Syntheses (70 a) and used for the Grignard reagent. The final toluene had b. p. 108–109° C, $n_D^{20.3} = 1.4963$. Activity yield 31 %.

Toluene-2-t II. o-Bromotoluene (m. p. —29––32° C, b. p. 177–180° C, $n_D^{19.0} = 1.5571$) was prepared in the same way and from the same o-toluidine as for toluene-2-t I, but in another batch. The toluene had b. p. 109.6–110.2° C, $n_D^{19.0} = 1.4968$. Activity yield 32 %.

Toluene-3-t. m-Bromotoluene (m. p. —37––35° C, b. p. 179–183° C, $n_D^{20.8} = 1.5532$) was prepared from p-toluidine (Kebo) via p-acetotoluide, 3-bromo-4-acetaminotoluene, and 3-bromo-4-aminotoluene according to Organic Syntheses (70 b). The m-bromotoluene was used for the Grignard reagent, and the final toluene had b. p. 109.0–109.5° C, $n_D^{20.5} = 1.4965$. Activity yield not measured.

Toluene-4-t. p-Bromotoluene (m.p. 24–26° C, b.p. 183–185° C) was prepared from p-toluidine (Kebo) according to Organic Syntheses (70 c) and used for the Grignard reagent. The resulting toluene had b.p. 110.5–111.5° C, $n_D^{20.6} = 1.4963$. Activity yield 36 %. An appreciable amount of p,p′-bitolyl was found in the residue of the first distillation. After recrystallization in alcohol it melted at 118–120° C and the hydrogen content was found to be 7.90 % (theoretical 7.76 %). The tritium content was measured, see below.

Toluene-α-t. Benzyl chloride ("for scientific purposes," Apot. Örnen) was purified by means of vacuum distillation and used for the Grignard reagent. The toluene had b.p. 108.7–109.0° C, and was diluted with some inactive toluene. After the dilution $n_D^{20.3} = 1.4963$. Activity yield 21 %.

Naphthalene-α-t. α-Bromonaphthalene from Svenska Finkemikalier was used for the Grignard reagent, which was prepared according to Organic Syntheses (71) on the scale 1 : 5. After the ether and the benzene had been distilled off, the latter at reduced pressure, the residue was recrystallized from alcohol. The crystals were dried over calcium chloride at reduced pressure and the substance distilled at 101–102° C/21 mm. M.p. 79–80.5° C. Activity yield 21 %.

Bromobenzene-2-t I. o-Nitroaniline (Eastman Kodak) was converted into 1-bromo-2-nitrobenzene according to F. ULLMAN (72), (the solution of cuprous bromide was prepared according to Organic Syntheses (70 c)). The bromonitrobenzene was reduced to o-bromoaniline according to HOLLEMAN (73 a). Finally the description for o-bromotoluene of Organic Syntheses (70 a) was used to convert the o-bromoaniline into o-dibromobenzene, which boiled at 110–112° C/25 mm and had $n_D^{20.0} = 1.6114$. The last compound was used for the preparation of a solution of o-bromophenylmagnesium bromide. The resulting bromobenzene had b.p. 58–61° C/26 mm, $n_D^{19.3} = 1.5599$. The activity yield was as low as 7.1 %, and the large high-boiling residue showed that extensive side-reactions had taken place.

Bromobenzene-2-t II. o-Dibromobenzene (b.p. 104–105° C/17 mm, $n_D^{19.0} = 1.6110$) was prepared from the same starting material and in the same way as in the preceding case but in another batch. In the preparation of the o-bromophenylmagnesium bromide solution freshly turned magnesium was used, and the reaction started more easily than in the preceding case, in which commercial turnings were used. The bromobenzene had b.p. 52–54° C/19 mm, $n_D^{18.3} = 1.5602$. Activity yield 11.2 %.

Bromobenzene-3-t I. Nitrobenzene (Svenska Finkemikalier) was brominated according to Organic Syntheses (70 d). The m-bromonitrobenzene was converted into m-dibromobenzene via m-bromoaniline according to HOLLEMAN (73 a). The m-dibromobenzene boiled at 115–117° C/35 mm, $n_D^{19.0} = 1.6083$. It was used for the preparation of the m-bromophenylmagnesium bromide solution. The resulting bromobenzene had b.p. 61–64° C/34 mm, $n_D^{19.3} = 1.5595$. Activity yield 21 %.

Bromobenzene-3-t II. m-Bromoaniline (Eastman Kodak) was converted into m-dibromobenzene according to HOLLEMAN (73 b). It boiled at 106.0–106.2° C/24 mm, $n_D^{19.0} = 1.6084$. The resulting bromobenzene had b.p. 45–47° C/12–13 mm, $n_D^{20.0} = 1.5584$. Activity yield 33 %.

Bromobenzene-4-t I. p-Dibromobenzene was prepared by bromination of pure benzene with iodine as a catalyst (74). It had m.p. 86–88° C and was used for the preparation of p-bromophenylmagnesium bromide solution. The resulting bromobenzene boiled at 59–61° C/31 mm, $n_D^{18.5} = 1.5589$. Activity yield 21 %.

Bromobenzene-4-t II. The same p-dibromobenzene as in the preceding preparation was used but first recrystallized once more, after which it had m.p. 87–88.5° C. The bromobenzene had b.p. 59–61° C/33 mm, $n_D^{19.4} = 1.5593$. Activity yield 28 %.

11 e. Investigation of possible exchange in the preparation of tritium hydrocarbons. 0.5 ml of toluene-4-t was mixed with 3.0 ml of inactive cumene and the mixture treated with 60 % perchloric acid, water, concentrated hydrochloric acid, water, sodium carbonate solution, and finally with water again. It was dried with calcium chloride for two days.

The mixture was for a while refluxed in the small Podbielniak column with a piece of tile (as when the ether is distilled off in the preparation), then the same with sodium metal, after which it was distilled from the sodium. When the distillate volume was 1.3–1.4 ml and the boiling point 148° C, 1.0 ml of inactive toluene was added to the remaining cumene, and 1.7 ml more was distilled off. The boiling point was 145° C, and the following (0.2–0.3 ml) was taken for a determination of tritium.

The specific activity was at most 0.07, if there was any real activity at all. If for instance it is assumed that owing to catalytic or other influences the tritium is spread out uniformly over all aromatic nuclei, the following figures are obtained. 0.5 ml of toluene equals 4.7 millimoles, and 3 ml of cumene 21.5 millimoles. Hence the tritium content per aromatic nucleus would have sunk in the proportion 4.7 : 26.2. If 2.1 ml of the cumene remained at the addition of 1.0 ml of inactive toluene and the same was repeated, the tritium content per aromatic nucleus would have sunk again in the proportion 15.1 : 24.5 or totally 1 : 9.0. In the analysis of toluene the activity is averaged over 8 and in cumene 12 hydrogen atoms. Thus the measured specific activity would have been further decreased in the proportion 2 : 3. At the time of measurement, the specific activity of toluene-4-t was 36.5, and $\frac{2}{3} \cdot \frac{36.5}{9} = 2.7$. The real specific activity was 2.6 % of this or less.

p,p′-bitolyl was obtained as a by-product in the preparation of toluene-4-t. Its specific activity was found to be 0.31 or less than 0.9 % of that of the toluene at the same time. It might be due to impurities still present after the single recrystallization.

11 f. Preparation of benzene-t by exchange. Since no orientation is necessary in the introduction of tritium into benzene, the exchange reaction with aqueous sulphuric acid was used in some cases. According to INGOLD, RAISIN, and WILSON (41) an acid containing slightly more than 50 mole per cent H_2SO_4 was used. They found that acid of this concentration caused a fairly rapid exchange without too much sulphonation. At higher concentrations the latter becomes more predominant, and in the present investigation it has been found that in fuming sulphuric acid practically no exchange takes place before sulphonation. Of course still less occurs afterwards owing to the deactivating influence of the sulphonic acid group.

The amounts used were 4 ml of concentrated sulphuric acid, 1 ml of water (containing the tritium) and 12 ml of benzene. In this mixture about 74 % of the total hydrogen present belongs to the benzene. INGOLD, RAISIN, and WILSON found that deuterium is very nearly uniformly distributed in this system, the relative deuterium content being slightly higher in the hydrocarbon. Thus about three fourths of the tritium could be supposed to be organically bound at equilibrium. However, the magnetic stirring applied was certainly less effective than the mixing used by the authors cited, who obtained equilibrium with deuterium within 3 to 4 days at room temperature. In the present case 17 % of the tritium was recovered in the benzene after 5 days' stirring (benzene-t III), and a second treatment of the separated acid with 12 ml of fresh benzene gave about the same amount of benzene-t in 7 days (benzene-t IV).

The benzene-acid mixture was magnetically stirred in a flask with a ground stopper. After separation the benzene was washed with water, sodium carbonate solution, and water again. It was dried with calcium chloride and distilled twice from sodium metal at atmospheric pressure. Each time some inactive benzene was added to the distillation residue (which was equal to the hold up of the column) and distilled in order to carry most of the activity left. The physical data of the two benzene preparations are given below. The boiling points are uncorrected.

Benzene-t III. B.p. 78.5–79.2° C, $n_D^{18.6} = 1.5016$.

Benzene-t IV. B.p. 78.8–79.1° C, $n_D^{20.3} = 1.5006$.

11 g. The specific activity of the tritium preparations. In Table 11: I the specific activity of the compounds, the preparation of which was described above, is given. It was measured by the method described in the discussion of the tritium determination and is given in the units used throughout this paper, i.e. one sixty-fourth of the number of disintegrations per minute in the Geiger-Müller tube used at the

Table 11: I

Specific activity of tritium preparations.

Compound	Spec. act.	Number of parallel det.	Date
Benzene-t I	31.5	2	21.10.48
Benzene-t II	48.3	2	29.1.49
Benzene-t II a[1]	9.71	1	21.10.49
Benzene-t III	16.9	2	2.12.49
Benzene-t IV	15.9	1	23.12.49
Toluene-2-t I	33.4	1	13.9.48
Toluene-2-t II	46.8	1	27.1.50
Toluene-3-t	28.1	1	10.9.48
Toluene-4-t	37.3	2	30.3.48
Toluene-α-t	8.00	2	24.1.49
Naphthalene-α-t	24.1	1	4.2.49
Bromobenzene-2-t I	10.4	2	20.10.49
Bromobenzene-2-t II	24.7	2	2.12.49
Bromobenzene-3-t I	26.6	2	27.10.49
Bromobenzene-3-t II	70.3	1	20.1.50
Bromobenzene-4-t I	92	2	1.9.49
Bromobenzene-4-t II	58.9	2	2.10.49

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[1] Benzene-t II a was made by dilution of benzene-t II with pure benzene.

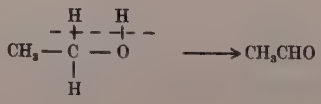
standard hydrogen filling pressure 90 mm of mercury. The unit corresponds to about 250 disintegrations per millimole hydrogen per minute. The number of parallel determinations is also given, and when parallel determinations were carried out on different days, they were made comparable by correction for the decay (the half-life was assumed to be exactly 12 years). The last column gives the date corresponding to the specific activity figure given.

11 h. An attempt to prepare nitrobenzene-3-t by reduction of m-nitrophenyldiazonium sulphate. As mentioned in the previous discussion an attempt was made to prepare nitrobenzene-3-t from m-nitroaniline by means of the reduction of the diazonium sulphate with ethyl alcohol containing tritium in the hydroxy group. The description of Organic Syntheses (70 e) for m-nitrotoluene from m-nitro-p-toluidine was followed in principle, but on the scale 1 : 22, e.g. 0.05 mole of the aromatic compound. This involves the use of 22.7 g of ethyl alcohol, 11.4 g of concentrated sulphuric acid, and 5.5 g of water to dissolve the sodium nitrite. If all hydrogen atoms of the water and the sulphuric acid, one of the alcohol molecule, and the two amino hydrogens of the amino group of the nitroaniline are considered exchangeable, the total amount of exchangeable hydrogen in the system is about 1.6 g-atoms. (The number of hydrogen atoms of the organic molecule taking part in the exchange is of minor importance since only 0.05 mole is present.)

The m-nitroaniline (m.p. 112–114° C) was prepared from m-dinitrobenzene (Kebo) according to ORTHNER-REICHEL (75). When the diazotization was completed but the icebath not removed, 35 mg of radioactive water was stirred into the mixture. The vigorous stirring was continued for some minutes before the warming began. After the gas evolution had ceased, the mixture was distilled through a glass bead column until the temperature of the vapours reached 80° C. Then the mixture was diluted with water and distilled with steam. The nitrobenzene was extracted completely from the distillate by means of benzene, which was finally distilled off from the nitrobenzene under reduced pressure. The remaining nitrobenzene weighed 1.9 g and was diluted with 1.1 g inactive nitrobenzene, after which all of the liquid was distilled at about 98° C/20.5 mm within less than one degree.

The specific activity (if any) of the resulting nitrobenzene was found to be at most 0.14, corresponding to 0.22 before the dilution. The radioactive water used (4 mg-atoms hydrogen) had the initial specific activity $2 \cdot 10^4$ and was diluted with protons 400 times, corresponding to a specific activity reduced to 50. In the analysis, the hydrogen of all five positions was finally mixed. If tritium and protium had had the same probability of reaction, a specific activity of 10 would have been found. The real value was 2 % of that or less.

A hydrogen loss in the different operations of the purification was hardly probable. The sulphuric acid was never highly concentrated after the dilution and the nitro group deactivates the benzene nucleus strongly. A very slow reaction of tritium as compared to protium might afford the explanation, but it seems more probable that the hydrogen of the oxy group does not enter the aromatic nucleus. In the formation of acetaldehyde also one of the methylene hydrogens is lost,



$$CH_3-\overset{H}{\underset{H}{C}}-\overset{H}{O} \longrightarrow CH_3CHO$$

and it may be this one that enters the nucleus. (The acetaldehyde formed will possibly exchange its hydrogen in the acid solution, but this would change the calculations but little because of the excess of water and alcohol.) From this it may be concluded that the alcohol reduction of diazonium salts cannot conveniently be used for the introduction of isotopic hydrogen into aromatic nuclei.

12. General principles of the substitution experiments

The outlines of the arrangement of experiments with isotopic hydrogen, especially with unweighable amounts of tritium, have already been given in paragraph 5. The more detailed experimental technique, common to the substitution experiments which will be described in the following paragraphs, and some conditions to be fulfilled for the experiments to be possible, will be reviewed in the present paragraph.

Since a hydrogen isotope is followed quantitatively, hydrogen exchange between different reactants has to be avoided. It caused no difficulty in the nitration and sulphonation experiments, in which it was easily proved to be absent, but it was not possible to avoid it in the catalytic bromination. Since aluminium chloride causes a rapid exchange of hydrogen between aromatic molecules and hydrogen chloride, it was not possible to make experiments on the mechanism of the Friedel-Crafts reaction, which requires a rather large amount of catalyst.

From paragraph 9 b it is evident that the yield of the reaction should be high in order to make the determination of the rate ratio as accurate as possible. Therefore only such reactions could be taken into consideration as lead to a fairly unitary product. Many mono-substitutions of ortho-para-directing benzene derivatives do not conform to this requirement, e.g. the nitration of toluene or bromobenzene. If the reaction is carried further to the di-derivative, however, the 2,4-dinitro derivatives are obtained in good yields.

Side-reactions will cause no trouble unless they are rapid enough to impair the yield too much. If reactions might occur, which preferred tritium to protium, the tritium would be carried away and too small a tritium concentration would then be found in the main product. This would give a false equality of the reaction rates in the main reaction, although it might prefer protium. However, this is hardly possible in the present case, because the hydrogen is initially held by a very strong chemical bond and must be loosened in all possible reactions.

The derivatives are generally obtained in a very impure state, and before the determination of tritium it is always necessary to purify them. Since it is much easier to purify a solid substance by recrystallization and control its purity by means of its melting point than to distil a liquid when only small amounts are available, reactions leading to solid products were always preferred.

Also in the purification of the product it is necessary that no hydrogen exchange takes place. All derivatives handled here contain deactivating substituents, and they were never exposed to strong donors of protons, so the danger was not great. The consistency of the results show that no irregular factor is involved.

It was considered almost axiomatic that no separation of isotopic molecules should take place in the recrystallizations. The difference in interaction with the surroundings between a common molecule and one containing one tritium atom among its hydrogens is certainly very small. In the case of m-dinitrobenzene an experiment with three successive recrystallizations was made, and the tritium concentration showed a slight decreasing tendency, but the differences were so small that they did not with certainty exceed the experimental error. Thus it is impossible to say if they correspond to a real separation or exchange effect, but in any case the effect is too small to influence the results appreciably. The numerical data of the experiment will be given in what follows.

The recrystallizations were never forced to give as high yields as possible because the purity of the product was the primary object. The most convenient way of determining the yield in the substitution reaction is the method of "isotopic dilution". At a moment when no considerable amounts have been lost as yet, a weighed amount of the corresponding pure compound, containing a known concentration of radioactive molecules of the same kind, was added to the bulk, which should be inactive in these experiments. The mixture is then dissolved and recrystallized in the common way. From the specific activity of the purified preparation, the weight of the initial bulk of inactive compound may be calculated, if no separation has occurred.

In the following paragraphs the experiments on nitration, bromination and sulphonation will be described. After the main reactions have been reported and their results given, control experiments are described, e.g. determination of yield, control of hydrogen exchange etc. The primary results of the measurements of the change in tritium content caused by the substitutions will be numerically treated in part V.

13. Experiments on nitration

The dinitrations of benzene, toluene, bromobenzene, and the mononitration of naphthalene were investigated. These reactions give a single solid product with a fairly high yield.

13 a. Dinitration of benzene. The nitrations 1–3 were carried out according to the following general method.

0.50 ml of benzene-t was shaken in a closed vessel for 4 minutes together with 5–8 ml of a mixture of 1 part by volume of concentrated nitric acid (sp. gr. 1.40) and 2 parts of concentrated (96–96.6 %) sulphuric acid. In some of the experiments 30 mg of water had been added to the acid in order to make the conditions similar to those existing when the hydrogen exchange was controlled and tritium-containing water was introduced in the same manner.

After the shaking the mixture was poured into cold water or on to ice. The precipitated crystalline solid was filtered off, washed with water, and recrystallized from alcohol. The m-dinitrobenzene was finally dried in vacuum over calcium chloride.

Experiment 4 was on a scale about three times larger than the preceding one. It was made in order to get a large amount of product, which could be recrystallized successively for the investigation of isotopic separation (see below).

In Table 13: I the detailed data about the individual experiments are given.

The hydrogen exchange during the reaction was not investigated, because the composition of the acid mixture was the same as in the nitration of toluene, where such an exchange was proved to be absent. The exchange is certainly slower with benzene than with toluene.

Determination of yield. An experiment exactly similar to exp. 2 above was carried out on inactive benzene. In order that no benzene should evaporate during the mixing, a flask with a small separate container for the benzene was used. Thus the flask could be stoppered before the reagents came into contact with each other and

Table 13: I

m-Dinitration of benzene-t.

The symbols y and y_0 have the same meaning as in paragraphs 9 a b; y has been corrected to the same point of time as y_0, cf. Table 11: I.

Exp.	Benzene prep.	Benzene ml	Mixed acids ml	Water ml	M. p. of dinitrobenzene (uncorr.)	Spec. act.	Date	y/y_0
1	Benzene-t I	0.50	5	—	86.5—89° C	30.9	29.11.48	0.658
2	» II	0.50	8	0.03	86—88° C	47.9	2.3.49	0.664
3	» III	0.50	8	0.03	87.5—89° C	16.8	17.12.49	0.663
4	» III	1.50	30	0.2	88—90° C	17.0	8.12.49	0.671

Mean value $y/y_0 = 0.664 \pm 0.007$.

the evolution of heat began. 108.1 mg m-dinitrobenzene-t was added to the washed, crude product, and the whole was recrystallized in alcohol. The melting point of the product was 87.5-89° C, and the specific activity had decreased from 16.8 to 2.16. Hence the weight of the m-dinitrobenzene in the crude product was $\left(\frac{16.8}{2.16} - 1\right) \cdot 108.1$ mg and since the pipette gave 431 mg of benzene, the yield was found to be 79 %. The relative error will be of about the same magnitude as in Table 10: I.

In another experiment the active compound had already been added to the benzene in the separate container and consequently was present during the nitration. The result was 86 % yield. It might be feared that some of the active compound could be converted into the trinitro compound, since it was present for a longer period than the compound formed. On the other hand, the introduction of a third nitro group into benzene is a very slow reaction, and the discrepancy is more likely to be due to incomplete precipitation of the crude product in the first experiment. Probably the yield is at least above 80 %.

The separation of isotopic molecules in the recrystallization was investigated in the following way. The product from experiment 4 was recrystallized two times more in the common proportion of alcohol (here 10–20 ml). The specific activity of the first crystals was 17.0 (this value was used in Table 13: I), and the corresponding values of the others were 16.8 and 16.4. Thus the concentration of molecules containing tritium seemed to be slightly decreasing. However, the total difference was not larger than 3.6 % and the whole effect may be illusory. According to the values y/y_0 in Table 13: I the value 17.0 is also unusually high. It might be concluded that no separation effect (or exchange with the alcohol) of importance occurs.

13 b. Dinitration of toluene. When toluene is nitrated with the same mixture of nitric and sulphuric acid as was used in the case of benzene, a good yield of 2,4-dinitrotoluene is obtained.

The general procedure in the nitrations of toluene was the following:

0.50 ml of toluene-t was shaken in a closed vessel for 3 minutes with 5 or 10 ml of a mixture of 1 part by volume of concentrated nitric acid (sp. gr. 1.40) and 2 parts of concentrated (96–96.6 %) sulphuric acid. In some of the experiments a small amount of water was added to the acids.

Table 13: II

2,4-Dinitration of toluene-2-t.

For symbols etc., cf. the heading of Table 13: I.

Exp.	Toluene prep.	Mixed acids ml	Water ml	M. p. of dinitrotoluene (uncorr.)	Spec. act.	Date	y/y_0
1	Toluene-2-t I	5	—	68.5—70° C	23.1	18.9.48	0.519
2	» I	5	0.03	67 —69°5 C	22.2	24.9.48	0.498
3	» I	10	—	66.5—69° C	22.2	2.3.49	0.512
4	» II	5	0.03	67.5—69° C	32.2	27.1.50	0.516
5	» II	10	0.06	68.5—69°5 C	32.7	28.1.50	0.524

Mean value $y/y_0 = 0.514 \pm 0.016$.

After the shaking the mixture was poured on to ice. The crystalline solid was filtered off, washed with water, and recrystallized from alcohol. The 2,4-dinitrotoluene was dried over calcium chloride under reduced pressure.

The detailed data about the individual experiments on toluene-2-t are given in Table 13: II. When 10 ml of the mixed acids was used, the mixture looked homogeneous, but not with 5 ml.

The hydrogen exchange during the reaction was investigated in the following manner. 0.50 ml of inactive toluene was nitrated by means of 5 ml of the common mixed acids, to which 30.9 mg water containing tritium had been added. The acid contains about 260 mg-atoms of hydrogen, and the specific activity is decreased from $4.5 \cdot 10^3$ for the water to about 59. If only one of the hydrogen positions of the dinitrotoluene contains exchanged hydrogen, the specific activity is further decreased to one sixth in the analysis. Consequently a specific activity of about 10 would be obtained if the exchange had come to an equilibrium (the amount of hydrogen in the hydrocarbon may be neglected as compared to that of the acid mixture), for INGOLD et al. (41) have found that deuterium is nearly uniformly distributed between sulphuric acid and benzene. The specific activity was found to be at most 0.1 if there was any real activity at all. It must be concluded that there is no appreciable exchange under the conditions of the reaction. In any case tritium cannot leave the aromatic nucleus more easily than it enters it, for INGOLD et al. (41) found that deuterium slightly prefers the aromatic position to that in sulphuric acid.

Control experiments. In order to check the preparative methods, toluene-3-t, -4-t, and -α-t were nitrated as in exp. 1 above. The results are shown in Table 13: III. They are in agreement with the theory that requires the y/y_0 values 1, 0, and 1.

Table 13: III.

2,4-Dinitration of toluene-3-t, -4-t, and -α-t.

For symbols etc., cf. the heading of Table 13: I.

Exp.	Toluene prep.	M. p. of dinitrotoluene (uncorr.)	Spec. act.	Date	y/y_0
6	Toluene-3-t	68 —69° C	37.1	16.9.48	0.990
7	Toluene-4-t	67.5—68°5 C	1.06	20.9.48	0.022
8	Toluene-α-t	69 —70° C	10.5	18.2.49	0.984

Determination of yield. The yield was determined by the method of isotopic dilution. In two experiments inactive toluene was nitrated with the same mixture as in exp. 2 above. In one of them the washed crude product was mixed and recrystallized together with a pure sample containing 2,4-dinitrotoluene-3-t and -5-t. The yield was found to be 75 %. In the other experiment 2,4-dinitrotoluene-α-t was added at the end of the nitration and shaken with the nitration mixture for about a minute before the mixture was poured out on to ice. A yield of 80 % was found. The comments are the same as the corresponding ones in paragraph 13 a.

13 c. Dinitration of bromobenzene. In the case of bromobenzene it is possible to obtain the 2,4-dinitro derivative with a very good yield, since m-nitrobromobenzene is formed in a very slight amount in the first step.

The general nitration procedure was the following.

0.50 ml of bromobenzene was shaken in a closed vessel for 5 minutes with 10 ml of a mixture of equal parts by volume of concentrated nitric acid (sp. gr. 1.42) and fuming sulphuric acid (20–30 % SO_3), to which a small amount of water had been added in some of the experiments.

After the shaking the mixture was poured into ice water. The crude product was filtered off, washed with water, and recrystallized from alcohol. Finally the 2,4-dinitrobromobenzene was dried in vacuum over calcium chloride.

The detailed data about the individual experiments on bromobenzene-2-t are given in Table 13: IV.

Table 13: IV.

2,4-Dinitration of bromobenzene-2-t.

For symbols etc., cf. the heading of Table 13: I.

Exp.	Bromobenzene prep.	Water added ml	M. p. of dinitrobromobenzene (uncorr.)	Spec. act.	Date	y/y_0
1	Bromobenzene-2-t I	—	67 —69° C	8.43	28.10.49	0.487
2	» II	0.5	67 —70° C	19.8	5.12.49	0.481
3	» II	0.05	69.5—72° C	21.1	20.1.50	0.517

Mean value $y/y_0 = 0.495 \pm 0.022$.

The hydrogen exchange during the reaction was investigated in the usual manner. 0.50 ml inactive bromobenzene was nitrated with 10 ml of the acid mixture, to which 15.5 mg of water containing tritium and 34.5 mg of inactive water had been added. The tritium water originally had the specific activity $4.6 \cdot 10^4$. The mixture contains about 520 mg-atoms of hydrogen, and consequently the specific activity became about 150. If only one position of the final dinitrobromobenzene contains exchanged hydrogen, the specific activity is further decreased to one third in the analysis. The amount of hydrogen in the organic molecule may be neglected in the calculation of the dilution, and consequently a specific activity of 50 would have been obtained at equilibrium, if the tritium is equally distributed between the organic position and the acid. The actual value was less than 0.1 if there was any real activity at all. Thus exchange is of no importance in the experiments.

Control experiments. In order to check the method, the other bromobenzene-t isomerides were nitrated by the same mixture as used in exp. 3 above. The results are given in Table 13: V.

Table 13: V.

2,4-Dinitration of bromobenzene-3-t and -4-t.

For symbols etc., cf. the heading of Table 13: I.

Exp.	Bromobenzene prep.	M. p. of dinitrobromobenzene (uncorr.)	Spec. act.	Date	y/y_0
4	Bromobenzene-3-t I	67 —70° C	42.4	29.10.49	0.956
5	» II	71 —73°5 C	119	26.1.50	1.016
6	Bromobenzene-4-t I	67 —71° C	0.78	1.10.49	0.005
7	» II	67.5—70°5 C	0.23	29.10.49	0.002

The agreement with the theoretical figures 1 and 0 is rather good except in exp. 4. Bromobenzene-3-t I, however, was made in several more preparative steps than bromobenzene-3-t II, and the deviation is probably due to the occurrence of isomerides.

Determination of yield. Inactive bromobenzene was nitrated with the same mixture as was used in exp. 3 above. In one experiment 2,4-dinitrobromobenzene-6-t was added to the washed crude product, in another a mixture of 2,4-dinitrobromobenzene-3-t and -5-t was added to the bromobenzene from the beginning. The results were respectively 86 and 97 %. It seems a yield of 90 % could safely be assumed.

13 d. Nitration of naphthalene. When nitrated with concentrated nitric acid, naphthalene gives a good yield of α-nitronaphthalene. Naphthalene-α-t was nitrated according to the following general method.

0.50 g of naphthalene-α-t was shaken in a closed vessel for 5 or 10 minutes with 5 or 10 ml of concentrated nitric acid (sp. gr. 1.40), to which 2 % by volume of water had been added. After the shaking the mixture was poured on to ice. The organic phase solidified easily, was filtered off, washed with water, and recrystallized from alcohol. It was finally dried in vacuum over calcium chloride.

The detailed data about the individual experiments as well as their results are shown in Table 13: VI.

Table 13: VI.

α-Nitration of naphthalene-α-t.

For symbols etc., cf. the heading of Table 13: I.
Of parallel determinations only the mean is given.

Exp.	Naphthalene prep.	Acid + water ml	Time of shaking min.	M. p. of α-nitronaphthalene (uncorr.)	Spec. act.	Date	y/y_0
1	Naphthalene-α-t	5	5	55 —56° C	21.6	4.2.49	0.784
2	»	5	5	57 —58° C	20.6	17.2.49	0.748
3	»	10	10	55.5—56°5 C	20.0	20.1.50	0.766

Mean value $y/y_0 = 0.766 \pm 0.018$.

In exp. 1 and 2 an appreciable evolution of heat began after about 3 minutes and an oily layer was formed instead of the naphthalene crystals. In exp. 3 the same happened after about 6 minutes.

The hydrogen exchange during the reaction was investigated as usual. 0.50 g of inactive naphthalene was nitrated with the same amount of acid as used in exps. 1 and 2. 23.6 mg of the water had an initial specific activity of $4.6 \cdot 10^4$. The acid contained about 350 mg-atoms of hydrogen, and its specific activity became about 350. If three positions of the nitronaphthalene are assumed to contain exchanged hydrogen, the specific activity is decreased in the ratio 3 : 7 in the analysis. With a uniform tritium distribution between the acid and these three positions, a specific activity of 150 should have been found (the amount of hydrogen in the naphthalene is neglected as compared to that of the acid). Actually less than 0.1 was found, if the nitronaphthalene contained any tritium at all. Thus no disturbing hydrogen exchange occurs.

Determination of yield. Inactive naphthalene was nitrated in the same manner as in exps. 1 and 2. Some α-nitronaphthalene-α′-t was added to the crude product. From the specific activity of the pure product, the yield 93 % was calculated.

In another experiment inactive naphthalene and a known amount of α-nitronaphthalene-α′-t were shaken together with nitric acid (sp. gr. 1.39) and 2 % by volume of water. The heat evolution began already after about 3 min., although it took a longer time with this weaker acid and pure naphthalene. Probably the melting point was depressed by the mixing of the two solids. The result was 95 % yield.

14. Experiments on bromination

p-Dibromobenzene was prepared from benzene-t with aluminium chloride, ferric bromide and iodine as catalysts. Comparative experiments were made on the different isomerides of bromobenzene-t.

14 a. Bromination with aluminium chloride as catalyst. Benzene-t was brominated according to the following general method.

2–35 mg of anhydrous aluminium chloride and 0.62–0.70 ml of bromine were added to 0.50 ml of benzene-t in a small flask with a reflux condenser during the course of $^1/_2$–1 minute. After some time, when practically all of the content had solidified, it was treated with a solution of sodium hydroxide, with hot water, generally also with dilute sulphuric acid, and water again. The crude product was recrystallized from alcohol. The final product was dried in vacuum over calcium chloride.

With too much bromine and a long time of reaction higher derivatives are easily formed.

The detailed data of the experiments are given in Table 14: I.

The product of exp. 2 was probably contaminated with higher bromine derivatives.

Comparative experiments. p-Bromination of the isomerides of bromobenzene-t, especially that of bromobenzene-4-t, revealed that extensive exchange of hydrogen accompanies the bromination. This could also have been anticipated from the results of KLIT and LANGSETH (42). The method was mainly the same as for benzene. 0.50 ml of bromobenzene and 0.26–0.30 ml of bromine were used. The detailed data are given in Table 14: II.

Table 14: I.

p-Dibromination of benzene-t ($AlCl_3$).

For symbols etc., cf. the heading of Table 13: I.

Exp.	Benzene prep.	$AlCl_3$ mg	Br_2 ml	Time of reaction	M. p. of p-dibromobenzene (uncorr.)	Spec. act.	Date	y/y_0
1	Benzene-t II	21.4	0.70	1 hour	84—87° C	58.0	10.3.49	0.806
2	» IIa	34.2	0.62	6 days	75—77° C	13.2	2.11.49	0.906
3	» III	2.3	0.62	1 day	85—88° C	19.7	12.1.50	0.781
4	» III	5.4	0.62	1 day	84—86° C	19.3	12.1.50	0.765

Table 14: II.

p-Bromination of bromobenzene-2-t, -3-t, and -4-t ($AlCl_3$).

For symbols etc., cf. the heading of Table 13: I.

Exp.	Bromobenzene prep.	$AlCl_3$ mg	Br_2 ml	Time of reaction	M. p. of p-dibromobenzene (uncorr.)	Spec. act.	Date	y/y_0
5	Bromobenzene-2-t I	30.7	0.26	1 day	84—86°.5 C	12.0	7.11.49	0.923
6	Bromobenzene-3-t I	33.8	0.26	1 day	84—86°.5 C	32.5	7.11.49	0.980
7	Bromobenzene-4-t I	29.0	0.30	19 hours	84—86° C	22.9	3.11.49	0.201
8	» I	26.7 +15.0[1]	0.30	5 hours	84—86° C	22.5	4.11.49	0.197
9	» I	41.2	0.30	5 hours	84—86° C	27.3	29.9.49	0.238
10	» II	26.2	0.30	3 days	81—84° C	34.9	3.10.49	0.474

[1] Added after 1 hour.

It was observed that bromine reacted less vigorously with these bromobenzene-t preparations than with commercial bromobenzene. Several attempts to purify the two kinds of bromobenzene did not affect this difference, which is thought to be due to some inhibitor formed in the Grignard reaction. However, from a comparison of the figures of y/y_0 with the theoretical values 1, 1, and 0 it is evident that extensive exchange takes place between the aromatic molecule and the hydrogen bromide before the latter leaves the mixture. The product of exp. 10 was certainly contaminated with higher bromination products.

14 b. Bromination with ferric bromide as catalyst. Some experiments were carried out with ferric bromide as catalyst in the bromination of benzene-t and bromobenzene-4-t. The results are rather similar to those in the previous paragraph.

The method was mainly the same as in the previous paragraph. 0.50 ml of the organic liquid was used in each experiment. The catalyst was added as iron powder (ferrum reductum). In one experiment (exp. 2) carbon disulphide was used as a solvent, which was evaporated in a stream of air after the treatment with sodium hydroxide, dilute sulphuric acid and water. The residue was recrystallized from alcohol as usual.

The detailed data are given in Table 14: III.

Table 14: III.

p-Dibromination of benzene-t and p-bromination of bromobenzene-4-t ($FeBr_3$).

For symbols etc., cf. the heading of Table 13: I.
Of parallel determinations only the mean is given.

Exp.	Preparation	Fe mg	Br_2 ml	CS_2 ml	Time of reaction	M. p. of p-dibromobenzene (uncorr.)	Spec. act.	Date	y/y_0
1	Benzene-t II	10.4	0.80	—	45 min.	85—87°5 C	56.5	11.3.49	0.785
2	» II	4.5	0.70	3.0	1 day	85—87° C	57.4	21.3.49	0.799
3	Bromobenzene-4-t I	10.5	0.30	—	45 min.	85—87° C	14.1	29.9.49	0.123

From the result of exp. 3 it is evident that hydrogen exchange occurs also in this case between the aromatic nucleus and the hydrogen bromide.

14 c. Bromination with iodine as catalyst. The experiments with iodine as catalyst were carried out mainly in the same manner as those with aluminium chloride (paragraph 14 a). Thus 0.50 ml of the aromatic compound and no solvent were used. The crude product was sometimes not treated with acid.

The detailed data about the individual experiments on benzene-t are shown in Table 14: IV.

Table 14: IV.

p-Dibromination of benzene-t (I_2).

For symbols etc., cf. the heading of Table 13: I.

Exp.	Benzene prep.	I_2 mg	Br_2 ml	Time of reaction	M. p. of p-dibromobenzene (uncorr.)	Spec. act.	Date	y/y_0
1	Benzene-t I	5.0 +3.0[1]	1.0 +0.5[1]	2 days	84 —88° C	31.5	10.2.49	0.679
2	» I	44	0.70	1 day	85.5—87°5 C	32.3	10.2.49	0.696
3	» III	40.0	0.70	3 days	86.0—87°5 C	18.1	27.1.50	0.722

[1] Added after 1 day.

Determination of yield. 0.431 g inactive benzene was brominated with 44.4 mg of iodine and 0.70 ml of bromine. After one day the mixture was washed with sodium hydroxide and hot water. This crude product was recrystallized together with a known amount of p-dibromobenzene-t. The specific activity of the final product indicated a yield of 84 %.

Comparative experiments were carried out on the three isomeric bromobenzene-t. As previously 0.50 ml of the aromatic substance was used in each experiment. The detailed data are given in Table 14: V.

Table 14: V.

p-Bromination of bromobenzene-2-t, -3-t, and -4-t (I_2).

For symbols etc., cf. the heading of Table 13: I.
Of parallel determinations only the mean is given.

Exp.	Bromobenzene prep.	I_2 mg	Br_2 ml	Time of reaction	M. p. of p-dibromo-benzene (uncorr.)	Spec. act.	Date	y/y_0
4	Bromobenzene-2-t I	58.8	0.26	2 days	84—86°5 C	13.2	2.11.49	1.015
5	Bromobenzene-3-t I	62.5	0.26	2 days	83—87° C	31.5	2.11.49	0.947
6	Bromobenzene-4-t I	36.0	0.30	1 day	85—87° C	5.28	1.9.49	0.046
7	» II	60.8	0.30	1 day	83—85° C	3.90	21.10.49	0.053

From the results in Table 14: V it is evident that hydrogen exchange occurs, but to a less extent than in the case of the metal halide catalysts (the reaction rates were comparable).

One experiment with competition between only two different kinds of molecules was made by means of bromobenzene-4-t. The kinetics of such experiments were outlined in paragraph 9 c.

3.00 ml of bromobenzene-4-t II was brominated with 228 mg of iodine and 1.00 ml of bromine. The amount of the latter reactant is insufficient for complete p-bromination. After two days the liquid part was decanted off, treated with sodium hydroxide and water, and finally distilled in vacuum. The main fraction boiled at 50–53° C/16 mm and had $n_D^{20.2} = 1.5622$. A determination of tritium showed that the specific activity of the bromobenzene had increased with 54 %.

The crystals, too, were treated with sodium hydroxide and water. They were recrystallized together with the distillation residue, which was also crystalline at room temperature. In the recrystallization as small an amount of alcohol as possible was used, and it was tried to purify the material with a minimum of losses. The purified and dried product weighed 2.84 g, corresponding to a yield of 42 % (based on the bromobenzene). It is believed that the true yield was 50–60 %. The magnitude y/y_0 was found to be 0.034.

The last results will be discussed in a later paragraph.

15. Experiments on sulphonation

The sulphonation of benzene-t and bromobenzene-4-t was investigated. The sulphonation of the latter is directed almost exclusively into the para position, and the competition is one between only two kinds of molecules (paragraph 9 c).

15 a. Sulphonation of benzene. Benzene-t was sulphonated according to the following procedure:

0.50 or 1.00 ml of benzene-t was carefully added to about double the volume of fuming sulphuric acid (10–12 % SO_3), to which a few mg of water had been added in advance. The flask was stoppered and cooled with water for about half a minute, after which it was shaken for 8–15 min., in the beginning with cooling. The contents were poured into 5 ml of an ice-cooled, saturated solution of sodium chloride. The flask was washed with a few ml of the same kind of solution and this part added to the main solution. The precipitation of sodium benzenesulphonate was completed in a refrigerator, the crystals filtered off and washed with the solution of sodium chloride. After the crystalline mass had been sucked off, it was boiled three times with about 60 ml of absolute alcohol for the extraction of the sulphonate. The extract was evaporated and the dry residue dissolved in absolute alcohol. The solution was filtered and evaporated until crystallization began. The crystallization was completed in a refrigerator. The crystals were sucked off, boiled twice with ether, and dried at 110° C. Sometimes the recrystallization from alcohol and the washing with ether were carried out in the inverted order.

The product contained very little sodium chloride. Its composition was sometimes checked by means of a determination of hydrogen, which was made in the same way as the combustion for the determination of tritium. In this case, however, the water was absorbed in anhydrous magnesium perchlorate.

The detailed data of the experiments are reported in Table 15: I. The theoretical hydrogen content is 2.80 %.

Table 15: I.

Sulphonation of benzene-t.

For symbols etc., cf. the heading of Table 13: I. The yield refers to the final product; of paralle tritium determinations only the mean is given.

Exp.	Benzene prep.	Benzene ml	Acid ml	Water mg	Time of reaction min	Yield %	Hydrogen cont. %	Spe. act.	Date	y/y_0
1	Benzene III	0.50	0.91	—	8	27		18.1	13.1.50	0.897
2	» III	0.50	0.91	5.1	10	39	2.79	18.1	17.1.50	0.897
3	» IV	1.00	2.00	—	15	43	2.75	17.3	21.1.50	0.912
4	» IV	1.00	2.00	—	10	53		17.6	28.1.50	0.928

The hydrogen exchange during the reaction was investigated in the usual manner. 0.50 ml of inactive benzene was sulphonated as in exp. 1 with 0.91 ml acid, which had been mixed with 3.4 mg of radioactive water. The initial specific activity of the water was $3.9 \cdot 10^4$. The reaction mixture contained in all 64 mg-atoms of hydrogen. The average specific activity would be about 230. If only two of the hydrogen atoms of the product contained exchanged hydrogen, the specific activity would be decreased by a factor 2/5 in the determination, but on the other hand the specific activity of the hydrogen would be higher. In any case an equal tritium distribution between the acid and two positions in the product would have given a specific activity higher than 90. Actually 0.61 was obtained, indicating a very slow exchange without importance. Since about half the amount of hydrogen in the system belongs to the acid, it is probable that an exchange would tend to decrease the tritium content of the product, i.e. decrease the effect established.

The yield. Certainly most of the loss in the purification is due to incomplete precipitation of the sodium benzenesulphonate in the sodium chloride solution. Therefore it was tried to determine the yield before that step. Since anhydrous benzenesulphonic acid is difficult to work with, sodium benzenesulphonate-t was added after 10 minutes to the reaction mixture of an experiment with inactive benzene similar to exp. 3. When the reaction was interrupted 5 minutes later, practically all of the salt had dissolved. The specific activity of the final product indicated 106 % yield. This abnormal result indicates that a lower percentage of the added compound than of the bulk had ended in the product. It seems not impossible that the salt added, which contained the sulphonic acid as anion, was especially apt to form disulphonic acid, or perhaps to be desulphonated (31, 32). No conclusions about the yield can be drawn from this experiment.

15 b. Sulphonation of bromobenzene. In one experiment bromobenzene-4-t was sulphonated and the reaction interrupted at an intermediate stage. If the p-sulphonation, which is the only one proceeding with an appreciable velocity, is more rapid for protium than for tritium positions, the remaining part of the bromobenzene will have its concentration of tritium increased (paragraph 9 c). The following method was used:

3.00 ml of bromobenzene-4-t II and 3.00 ml of fuming sulphuric acid (10–12 % SO_3) were shaken together for 2 minutes with cooling of the flask in the beginning. The mixture was poured on to ice. The remaining bromobenzene was separated off and treated with a dilute solution of sodium carbonate. The bromobenzene was finally dried over calcium chloride and transferred to a distillation apparatus. In order to decrease the loss, ether was used to carry the residual parts and was evaporated afterwards. The bromobenzene was distilled at 42° C/11–12 mm within an interval of 0.2°. $n_D^{19.0} = 1.5604$. The weight of the distillate was 0.75 g or 17 % of the initial amount of bromobenzene. Its specific activity had been increased with 50 %.

The hydrogen exchange during the reaction. Exactly the same process was repeated with inactive bromobenzene and 7.7 mg of radioactive water added to the acid before the reactants were mixed. The remaining bromobenzene boiled at 41.5° C/11 mm within 0.2°. $n_D^{19.5} = 1.5596$. The weight of the distillate was 0.93 g, or 21 % of the initial amount of bromobenzene.

The reaction system contained about 250 mg-atoms of hydrogen in all, and the initial specific activity of the added water being $3.9 \cdot 10^4$, the average specific activity of the hydrogen of the system became about 130. If three atoms of the bromobenzene take part in the exchange, the specific activity is further decreased in the proportion 3/5 in the analysis. If the distribution of tritium between these positions and the acid had been uniform, the final specific activity would have been higher than 78, for the figure 130 was calculated as the average for all hydrogens and represents a lower limit. The actual value was 0.52. Thus the exchange was too slow to be of importance.

V. Discussion of Results

16. Nitration

16 a. The influence of side-reactions. In the experimental part the fraction of tritium retained in some competitive reactions has been determined. Within the limit of accuracy the results equal the fraction $\frac{n-1}{n}$, where n denotes the number of positions accessible to substitution. Comparison with paragraph 9 b shows that the rates of tritium and protium substitutions are roughly equal. It is of great interest to know the accuracy with which the ratio k_T/k_H may be put equal to unity according to the results. When the reaction is simple as in the case of naphthalene the accuracy in the determination of this ratio is easily found by means of the methods of paragraph 9 b. When the final compound is a product of subsequent reactions, special treatment is required.

In most cases the theoretical maximum yield in the substitutions is less than 100 % owing to parallel reactions. Nitrobenzene, for instance, gives less than 92 per cent of m-dinitrobenzene when nitrated at 40° C in sulphuric acid (6 a). If it is assumed in the calculations that the amount not used in the formation of the derivative desired remains unconsumed, the computed lower limit of k_T/k_H becomes somewhat lower than necessary. The reason is easily seen.

The side reactions may be assumed not to discriminate between tritium and protium or to prefer the latter. They cannot, however, be assumed to prefer tritium. Consequently they tend to increase the tritium concentration in the part not yet consumed or at least to leave it unchanged. The tritium content of the main derivative will then be increased or left unchanged by their presence.

If the side-reactions do not discriminate between the two kinds of molecules, they will at every moment consume material of the actual composition. If the main reaction prefers protium, the concentration of tritium will rise during the reaction, and the side reactions will consume material with very different tritium concentration at the beginning and at the end of the reaction.

Without any regard to the side reactions in the computation the result will be the same as if the main reaction were completed before the side-reactions began. In such an imaginary system, the side-reactions would have to consume what is left by the main reaction. If the latter is assumed to prefer protium, the former reactions are forced to consume more tritium than in the case of parallel reactions. Thus the amount of tritium in the main product is more likely to be increased in the real reaction than in the imaginary one, on which the calculation is based. Consequently the evaluation of the lower limit of k_T/k_H is based on an imaginary reaction, which is less sensitive to low values of k_T/k_H than the real one. The lower limit will therefore be a little too low.

A more exact treatment has shown, however, that the neglect does not increase the deviation from unity very much, and for the sake of simplicity the error will generally be calculated as if the amount not consumed by the main reaction were left unconsumed.

16 b. Dinitration of benzene-t. The reaction may be represented in the following way:

$$C_6H_6 \underset{x_1}{\rightarrow} C_6H_5NO_2 \underset{x_2}{\rightarrow} m\text{-}C_6H_4\,(NO_2)_2$$

where the amounts of reaction are represented below the arrows. The fraction of tritium retained in the first step is given by equation (9 : 9) as $f_6(x_1, \beta_1)$, where the index of f denotes the value of n. In the second step only two of the positions are accessible to substitution. Isotopic hydrogen may safely be assumed to have no directing influence. Consequently the tritium is uniformly distributed among five positions in the nitrobenzene, and the fraction retained in the second step becomes $\frac{1}{5}\,[3+2\,f_2(x_2, \beta_2)]$. According to Table 13: 1 the overall fraction found was 0.664±

± 0.007. The upper limit may safely be put equal to 0.68. With the use of this and an overall yield equal to 81 %, the following equations are obtained:

$$\tfrac{1}{5} f_6(x_1, \beta_1)\,[3 + 2 f_2(x_2, \beta_2)] = 0.68 \tag{16: 1}$$

$$x_1 x_2 = 0.81 \tag{16: 2}$$

When the product is the result of subsequent reactions, it is impossible to give both the upper and the lower limits of the β-values, because it is impossible to assign an arbitrary lower limit to any of them. The upper limit 1, however, may safely be assigned to any of them, and it is then possible to calculate the lower limit of the other, if reasonable values are assigned to the amounts of reaction. That is why the upper limit of the retained tritium fraction was used.

The second reaction is known to be slower than the first, and x_1 will at least be equal to x_2.

In the computation of the lower limit of β_1, the following values are used

$$x_1 = x_2 = \sqrt{0.81} = 0.9; \quad \beta_2 = 1$$

Equation (16: 1) gives

$$f_6(0.9, \beta_1) = 0.85$$

and the deviation of β_1 from unity is found by means of equation (9: 20):

$$\Delta \beta_1 = -\tfrac{36}{5}\,\varphi(0.9)\,[0.85 - \tfrac{5}{6}] = -0.16$$

The lower limit is $\beta_1 = 0.84$.

In the corresponding computation of the lower limit of β_2 the values $x_2 = 0.81$, $\beta_1 = 1$ are used. Equation (16: 1) gives

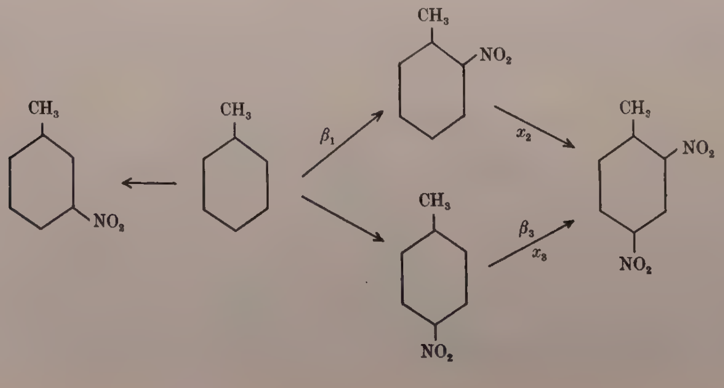
$$f_2(0.81, \beta_2) = 0.540$$

and equation (9: 20)

$$\Delta \beta_2 = -\tfrac{4}{1}\,\varphi(0.81)\,[0.540 - \tfrac{1}{2}] = -0.26$$

The lower limit is $\beta_2 = 0.74$.

16 c. Dinitration of toluene-2-t. The reaction scheme is rather complicated. It may be represented approximately in the following way:



The proportions o : m : p depend on the conditions. Too high a proportion of m will make the results too favourable. It seems 0.62 : 0.04 : 0.34 may be used in these approximate calculations (76).

For simplicity it will be assumed that the first nitration step is quantitative. The inactive molecules are then distributed as above and the initially radioactive ones in the ratio 0.31 $(1+\beta_1)$: 0.04 : 0.34, where β_1 represents the ratio k_T/k_H in the orthonitration. The quantity y/y_0 becomes for the ortho position

$$\frac{0.31}{0.69 + 0.31\,\beta_1} \cdot \frac{1}{0.62} = \frac{1}{2\,(0.69 + 0.31\,\beta_1)}$$

and for the para position

$$\frac{0.34}{0.69 + 0.31\,\beta_1} \cdot \frac{1}{0.34} = \frac{1}{0.69 + 0.31\,\beta_1}$$

If the yield in the p-nitration of the o-nitrotoluene is x_2 and that in the o-nitration of the p-nitrotoluene is x_3, the following equations are easily obtained from the experimental data:

$$\frac{0.62\,x_2}{2\,(0.69 + 0.31\,\beta_1)} + \frac{0.34\,x_3\,f_2\,(x_3,\,\beta_3)}{0.69 + 0.31\,\beta_1} = 0.76 \cdot 0.525 \tag{16: 3}$$

$$0.62\,x_2 + 0.34\,x_3 = 0.76 \tag{16: 4}$$

The yield has been taken as 76 %. The mean value of y/y_0 was given as 0.514 ± 0.016 in Table 13: II, but no experimental value exceeded 0.524, and therefore it seems possible to use a value as low as 0.525 here. β_3 refers to the value of k_T/k_H in the nitration of 4-nitrotoluene-2-t.

Equation (16: 3) may be written

$$0.31\,x_2 + 0.34\,x_3\,f_2\,(x_3,\,\beta_3) = 0.76 \cdot 0.525\,(0.69 + 0.31\,\beta_1) \tag{16: 5}$$

The lower limit of β_1 is obtained for f as small as possible or equal to 1/2. Independently of the ratio $x_2 : x_3$ the limit is found to be $\beta_1 = 0.85$.

On the other hand, the lower limit of β_3 is found when β_1 is as high as possible, i.e. equal to unity. The equations (16: 4) and (16: 5) then give

$$0.34\,x_3\,[f_2\,(x_3,\,\beta_3) - \tfrac{1}{2}] = 0.019$$

It is evident that the lower limit of β_3 is obtained when f is as large and x_3 as small as possible, or when $x_2 = 1$. Then $x_3 = 0.41$, and $f_2\,(0.41,\,\beta_3) = 0.636$. This corresponds to a point above the curve for $\beta = 0$ as seen from Fig. 9: 1. Thus no conclusions can be drawn about the vale of β_3 in this case, which, however, is a rather improbable one.

More probably the yield x_3 is at least as high as x_2 (76). In such a case $x_2 = x_3 = 0.792$. As before β_3 has its lower limit when $\beta_1 = 1$, and then $f_2\,(0.792,\,\beta_3) = 0.571$. Equation 9: 20 gives $\varDelta\beta_3 = -0.48$. The lower limit is 0.52, a result which is not very good, but which makes it probable that k_T and k_H are of the same order of magnitude also in this reaction.

Finally it may be of interest to calculate β_1 and β_3 from the assumption that $x_2 = x_3 = 0.792$ and $\beta_1 = \beta_3 = \beta$. The equation (16: 5) will then contain both β and $f(0.792, \beta)$. It is possible to get an approximate solution of this transcendental equation if the Taylor expansion of equation (9: 23) is substituted for f. The result is $\beta_1 = \beta_3 = 0.88$. Also this result makes it highly probably that both reactions proceed with approximately the same velocity in protium and tritium positions.

16 d. Dinitration of bromobenzene-2-t. The reaction scheme is less complicated than with toluene, because the amount of m-nitrobromobenzene formed in the first step is very small, not above 0.3 %. It will be neglected in the following treatment. The relative amounts of the other products depend somewhat on the conditions (6 b), but it seems the ratio o : p may be put equal to 0.34 : 0.66. As with toluene it will be assumed that the first nitration step is quantitative. The following equations are found in a manner analogous to that of the preceding paragraph:

$$\frac{0.34\, x_2}{2\,(0.83 + 0.17\,\beta_1)} + \frac{0.66\, x_3 f_2\,(x_3, \beta_3)}{0.83 + 0.17\,\beta_1} = 0.90 \cdot 0.52 \qquad (16{:}\ 6)$$

$$0.34\, x_2 + 0.66\, x_3 = 0.90 \qquad (16{:}\ 7)$$

The yield has been put equal to 90 %, a figure which seems reasonable in view of the experimental results. The mean value of y/y_0 was 0.495 ± 0.022 according to Table 13: IV, and it seems reasonable to use 0.52 as the upper limit.

The lower limit of β_1 with $\beta_3 = 1$ becomes 0.78 irrespective of the ratio $x_2 : x_3$. The lower limit of β_3 with $\beta_1 = 1$ and $x_2 = 1$ becomes 0.81. Thus in this case there seems to be little doubt that the o-nitration of bromobenzene and p-nitrobromobenzene both proceed with nearly the same velocity for protium and tritium.

16 e. Nitration of naphthalene-α-t. In this simple reaction it is possible to obtain both an upper and a lower limit of β. According to Table 13: VI the experimental limiting values of y/y_0 were 0.748 and 0.784. Since only three determinations in all exist, it is advisable to use the value 0.750 ± 0.035. The yield was found to be at least 93 %. Equation (9: 20) gives:

$$\Delta\beta = \mp \tfrac{16}{34}\,\varphi\,(0.93) \cdot 0.035 = \mp\, 0.23$$

The value of β is 1.00 ± 0.23. As Fig. 9: 4, curve C, shows, the tangent coincides very well with the curve for deviations from $\beta = 1$ as small as 0.23. The calculated error becomes slightly too small at the upper limit of β, but at the lower limit, which is of more interest, the calculated error becomes slightly too large.

16 f. The probable mechanism of nitration. In the preceding paragraphs the lower limits shown in Table 16: I have been calculated for the ratio k_T/k_H from the experimental data. It has to be noted that values joined with a bracket cannot be valid simultaneously, because each value requires its companion to be unity. The calculations are based on very unfavourable and even improbable presumptions. As shown in the case of toluene, more probable assumptions improve the figures considerably, and the value of p-nitrotoluene should certainly be comparable to the others.

Table 16: I.

Lower limits of k_T/k_H for nitrations at about 350° K.

Nitration of	Position	Lower limit of k_T/k_H
Benzene		0.84
Nitrobenzene	3	0.74
Toluene	2	0.85
4-Nitrotoluene	2	0
Bromobenzene	2	0.78
4-Nitrobromobenzene	2	0.81
Naphthalene	α	0.77

There is nothing in the experimental results that makes the value unity improbable in any of the reactions. The mean value in the case of toluene (Table 13: II) is somewhat high, but isomeric contaminations are likely to cause some deviations. Thus it is symptomatic that the best values are obtained with benzene-t as starting material, in which case this source of error is absent.

Only in the case of naphthalene an upper limit has been given. It is, however, highly improbable that the ratio should exceed unity, and such a limit offers little of interest.

The substitutions investigated differ in type. Benzene and naphthalene are pure aromatic hydrocarbons. The simple derivatives, nitrobenzene, toluene, and bromobenzene belong to three different types, respectively —I—T, +I, and —I+T, as they are generally denoted by means of Ingold's symbolism (cf. paragraph 2 d). Toluene has an activated nucleus, nitrobenzene and bromobenzene have theirs deactivated. In all these cases the ratio k_T/k_H has been found to be close to unity.

In view of the theoretical considerations of the present paper there seems to be little doubt that the hydrogen-loss in nitration is kinetically insignificant. It seems the two-step mechanism of paragraph 4 e is the only explanation, because it is the only simple way of realizing a rate-determining step not influenced by the mass of the hydrogen. The result, however, implies only that the hydrogen cannot be appreciably loosened in the activated state of the addition. From that fact, in turn, it seems natural to assume the existence of a relatively stable intermediate of the type pictured in Fig. 4: 1, for otherwise it would be hard to understand how, in the imaginary reversed process, it were possible to bring a hydrogen nucleus up to its normal bond distance from the carbon without reaching the potential energy maximum (from the other side). It seems natural to assume that the intermediate corresponds to a real minimum in the energy surface, and that saddle points on each side correspond to the losses of NO_2^+ or H^+. The first saddle point is at the highest level.

It is interesting to note that Ingold et al. (38) have recently arrived at the same conclusion as to the kinetical insignificance of the proton-loss. They have studied the influence of the solvent on the rate of reaction. Thus two completely independent ways of investigation have given the same result. The result, however, is in opposition to that of Bennett et al. (37), who found that the concentrations of hydrogen acceptors enter the expression of the reaction rate, i.e. that the proton-loss is rate-determining. Ingold et al. (38) give another explanation of the latter results.

17. Bromination

With all three catalysts used hydrogen exchange occurs. This is clearly shown by the experiments on bromobenzene-4-t, which should give a tritium-free product if no exchange occurred. The results cannot be due to isomeric contaminations in the preparations used, for the same preparations gave practically tritium-free 2,4-dinitrobromobenzene (Table 13: V).

The fact that the p-dibromobenzene always has its tritium content increased by the exchange agrees with the observation of KLIT and LANGSETH, who observed that deuterium prefers the position in benzene to that in hydrogen chloride (42). The effect should be still more pronounced with tritium and hydrogen bromide, the latter because the bond in hydrogen bromide is considerably weaker than that in hydrogen chloride.

The experiments with bromobenzene-4-t show that the exchange is less extensive with iodine as catalyst, than with the metal halides. This seems to hold irrespective of the reaction rates and the amounts of catalyst. Probably it is related to the strength of the acids present. It is remarkable that the tritium content of the p-dibromobenzene prepared from benzene-t with iodine as catalyst is smaller than when the other catalysts were used in the same reaction. The catalysts are present in small amounts and have little or no influence on the exchange equilibrium. Thus it seems certain that the tritium content in the absence of exchange would be less than any of the experimental figures, and more close to the results with iodine than to the others.

In fact, the values of y/y_0 in Table 14: IV are only slightly higher than 2/3, which is the theoretical figure for equal substitution rates of protium and tritium. The yield has been determined in an experiment closely analogous to experiment 2, and it was found to be 84 %. From this figure and a value of y/y_0 of about 0.70 the lower limit of the rate ratio in the bromination of benzene may be calculated. This first step of the formation of p-dibromobenzene leaves the fraction $f_6(x_1, \beta_1)$ of the tritium in the bromobenzene. x_1 and β_1 denote the yield and the ratio k_T/k_H in the first step. The tritium is uniformly distributed among the five positions. Irrespective of the ratio k_T/k_H in the second substitution, a fifth of the tritium is lost. Thus the following equation holds:

$$\tfrac{4}{5} f_6(x_1, \beta_1) = 0.70$$

The yield is certainly at least as high in the first step as in the second, hence $x_1 = \sqrt{0.84} \approx 0.92$. In the usual way the lower limit of β_1 is found to be 0.61. The true value of k_T/k_H is certainly higher, because the figure 0.70 includes the effect of exchange.

Thus it seems probable that bromination with iodine as catalyst follows the same course of reaction as nitration. There is no immediate reason why the catalyst should determine this part of the mechanism, and consequently it might be supposed that the same holds also for the other brominations. If the intermediate with the nitro group has some degree of stability, there is reason to think that the same is applicable to the corresponding ion with bromine.

The result of the competition between bromobenzene and bromobenzene-4-t might be thought to be astonishing in connexion with this picture of the mechanism. The specific activity of the initial substance was increased 1.54 times for an amount of reaction about 0.5–0.6. If no hydrogen exchange were operative, this would correspond to a k_T/k_H ratio of 0.37–0.53, cf. Fig. 9: 7. The values seem to be too high to allow for another type of mechanism, especially since it is known that at least some of the increase was caused by exchange.

More probably the whole increase is due to hydrogen exchange. The bromination under the same conditions is directed almost exclusively into the para position. There are reasons to think that also the exchange, which is another electrophilic substitution, would be directed strongly into the para position. Thus the tritium expelled in the bromination of some of the molecules would enter the para positions of the remaining molecules much more frequently than the other positions. In the final p-dibromobenzene only the tritium of the latter positions is retained. That is why the exchange seems to cause a more powerful effect in the incomplete reaction than in the common experiments.

A kinetical insignificance of the proton-loss agrees with the result of ROBERTSON et al. (77) who find that bases do not operate as catalysts on bromination. Generally the kinetical expressions are of very high order in respect to bromine or other brominating entities, as found by the school of ROBERTSON (27), by PRICE and ARNTZEN (78) and by BRADFIELD, DAVIES, and LONG (79). BREZHNEVA and ROGINSKY (80) interpret this as an inhibitory effect of the products. Generally it is interpreted in other ways.

Two types of explanation or a combination of them seem to be possible. In one of them the formation of the attacking entity is assumed to be very complex and require a high order of reaction. That has no bearing on the present results, which do not require an attack by a simple cation. According to the other, complex intermediates are formed between the aromatic molecule and bromine. Generally they are thought to be converted into the products under the influence of a secondary attack by bromine or some other entity. These pictures of the mechanism are in agreement with the present results as far as the final rate-determining attack aims at a splitting of the complex in such a manner that positive bromine enters the nucleus, and not at a removal of the hydrogen. The scheme of ROBERTSON, DE LA MARE and JOHNSTON (81) seems to fulfil the last requirement (A denotes the aromatic molecule):

$$A + Br_2 \rightleftarrows A, Br_2; \quad A, Br_2 + Br_2 \rightarrow ABr^+ + Br_3^-$$

$$ABr^+ \rightarrow \text{products}.$$

The last step is supposed to be rapid, and the authors also point to the intermediates of PFEIFFER and WIZINGER (13) to which ABr^+ is analogous.

Thus it seems that the kinetical experience is not in opposition to the present results as far as it has a bearing on them at all.

18. Sulphonation

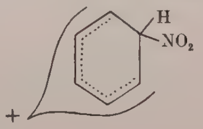
The experimental results on the sulphonation of benzene-t and bromobenzene-4-t indicate that tritium is substituted considerably more slowly than protium. It has been shown that hydrogen exchange does not affect these results. The exact value of k_T/k_H cannot be established without the true yield being known. The yields of the pure sodium benzenesulphonate shown in Table 15: I together with the values of y/y_0 of the same table correspond to points above the curve for $\beta = 0$ in Fig. 9: 3.

It is of interest, however, to observe that y/y_0 increases with the yield of pure product, which most probably runs parallel with the yield in the substitution process itself. In the case of bromobenzene-4-t the yield of the purified remaining initial material has very little to do with the true remaining amount.

In any case there is a pronounced difference between tritium and protium in the sulphonation of benzene and bromobenzene. As is evident from the parts II and III of the present paper there are two possibilities to explain this difference. The reaction might be either a pure S_E2 displacement or a two-step displacement with the second step rate-determining.

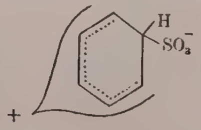
The results of the present investigation seem to indicate that the aromatic nitration and probably also the bromination proceeds via an intermediate of the type proposed originally by PFEIFFER and WIZINGER (13), and the existence of which has been admitted by several other authors. Even if a displacement of the pure S_E2 type cannot be excluded in the case of the sulphonation reaction, some interesting considerations arise if the intermediate mechanism is assumed to be realized even in the latter case. Then it becomes necessary to assume that the second step is rate-determining.

In nitration the intermediate will have the following constitution:



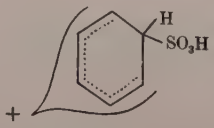
(A)

In sulphonation SO_3 is the most probable attacking entity, and the intermediate will be



(B)

This intermediate has been proposed by LANTZ (31), and it occurs also in a paper by BADDELEY, HOLT, and KENNER (32). Also if it is SO_3H^+ that attacks the benzene ring, it is probable that the same intermediate will be formed, because



(C)

will be a very strong acid owing to its possession of a positive charge (stronger than the ordinary sulphonic acids and sulphuric acid), and it is probable that the proton at oxygen is most rapidly expelled. This does not mean that (B), which is a tautomeric form of benzenesulphonic acid is necessarily more stable than the latter, but it is a question of reaction velocity. Thus the neutralization of nitromethane is much slower than that of a sulphonic acid, because the proton-loss from the much less ionic carbon-hydrogen bond is associated with an activation energy.

Now it seems natural that A should lose its hydrogen much more easily than B because of the difference in charge. Thus in the scheme of paragraph 4 f:

$$A + B + C + \cdots \underset{v_2}{\overset{v_1}{\rightleftarrows}} M + N + \cdots; \quad M + P + Q + \cdots \xrightarrow{v_3} Y + Z + \cdots$$

v_3 is certainly much smaller in the case of sulphonation than in the case of nitration. In the reverse reactions, NO_2 and SO_3 are probably lost, and since they carry no free charges, the velocity v_2 will not be influenced in the same way. Thus it would not be astonishing to find that $v_2 \gg v_3$ in the case of sulphonation, but $v_2 \ll v_3$ in the case of nitration. On the energy surface the saddle point corresponding to the proton-loss is at the highest level in sulphonation but that corresponding to the loss of NO_2^+ in nitration (cf. paragraph 16 f.). It is also possible that the proton removal in the case of sulphonation requires an acceptor though it does not in nitration.

One interesting consequence of the last-mentioned ideas would be the following. The kinetics of nitration would follow equation (4: 2), while the kinetics of sulphonation would follow (4: 4). Thus the orientation in the nitration of benzene derivatives is governed by the rate constant of the first step, but the sulphonation by the equilibrium constant of the formation of the intermediate and the rate constant of the second step. Differences in the quantitative directing power of substituents between the two kinds of reaction may at least to some extent be explained in this way.

The experimental results of this investigation and others are in agreement with the picture outlined.

In the investigations of HINSHELWOOD et al. on sulphonation by means of sulphur trioxide in nitrobenzene (23–25) it was found that two molecules of SO_3 take part in the rate-determining reaction. One explanation may be that the second removes the proton. In the case of sulphonation by sulphuric acid in nitrobenzene HINSHELWOOD et al. (39) propose SO_3H^+ as the sulphonating agent, and so do BRAND and RUTHERFORD (40) in the case of oleum, but COWDREY and DAVIES (82) point out that at least in oleum the physical difference between SO_3H^+ and SO_3 is vanishing owing to the hydrogen bond structure. The latter authors found that the activation energy in oleum is higher than that in sulphuric acid, probably because of the necessity of transferring the proton to a neutral acceptor in oleum instead of the anion HSO_4^- in the sulphuric acid.

The intermediate (B) as an intermediate in both sulphonation and desulphonation occurs in a paper by BADDELEY, HOLT, and KENNER (32), and it has been discussed especially by LANTZ (31). The reversibility of sulphonation, which has no equivalent in the other common substitutions is thought to be due to the negative charge of the sulphonic acid anion, which enables the attachment of a proton to the latter. Thus the desulphonation is supposed to proceed via the anion and the intermediate (B).

Finally, however, it should be pointed out once more that the results of the present investigation agree also with a pure S_E2 mechanism of sulphonation.

SUMMARY

The development and the present state of the theory of aromatic substitution has been reviewed. It has been pointed out that although the factors governing the ease and direction of the electrophilic attack on simple benzene derivatives are

rather well known, very little is known about the mechanism of the displacement of the hydrogen.

The different possible mechanisms of the replacement of the hydrogen have been discussed. There are mainly two alternatives, a pure S_E2 mechanism, and a two-step mechanism with an intermediate of quinonoid structure. It has been shown that isotopic hydrogen behaves differently in different alternatives. The arrangement of experiments with tritium in order to decide which of the alternatives corresponds to the true mechanism has been discussed.

The behaviour of different isotopes according to the theory of absolute reaction rates has been reviewed and the principles applied to the present problem. In a pure S_E2 mechanism and in the two-step mechanism with the second step rate-determining there will be a very pronounced difference in reaction rate between tritium and protium positions, but if the first step of the two-step mechanism is rate-determining, the isotopic mass of the hydrogen will have no influence.

The variation of isotopic concentrations in isotopic reactions has been treated mathematically and the formulae necessary for the computation of the ratio between the rates of tritium and protium substitutions from the results have been deduced.

The determination of tritium has been carried out according to a method already published by the present writer (59). The sources of error have been discussed and empirical material has been presented, according to which the accuracy in a single tritium determination on an organic substance is about ±2 %.

The preparative methods available for the introduction of isotopic hydrogen into organic molecules have been reviewed. Benzene-t, naphthalene-α-t, and the different isomerides of toluene-t and bromobenzene-t have been prepared in radiochemical quantities by means of the Grignard reaction. Benzene-t has also been prepared by exchange.

The following substitution experiments have been carried out: m-dinitration of benzene and 2,4-dinitration of toluene and bromobenzene with mixed sulphuric and nitric acids, α-nitration of naphthalene with nitric acid, p-dibromination of benzene and p-bromination of bromobenzene with aluminium chloride, ferric bromide and iodine as catalysts, sulphonation of benzene and p-sulphonation of bromobenzene with oleum.

From the results of the substitutions the lower limit of the ratio between the rates of tritium and protium substitutions has been calculated in the case of nitration, where the rates have been found to be equal within the accuracy of the method. The lower limits are such that it may be stated that the following nitrations take place according to the two-step mechanism with the first step rate-determining (the hydrogen-loss kinetically insignificant): nitration of benzene, m-nitration of nitrobenzene, o-nitration of toluene and bromobenzene, o-nitration of p-nitrobromobenzene, α-nitration of naphthalene, and probably also o-nitration of p-nitrotoluene.

In the case of bromination all catalysts have been found to cause hydrogen exchange between the liberated hydrogen bromide and the aromatic nucleus. The exchange is slowest in the case of iodine, and the results from the iodine-catalyzed bromination point to the same mechanism as for nitration.

In sulphonation the tritium has been found to be substituted more slowly than protium. It has not been possible to calculate the rate ratio owing to difficulties in the determination of the yield, but the difference is very pronounced. According to this result the sulphonation may be a pure S_E2 replacement or follow the two-step mechanism with the second step rate-determining. Although it is impossible to decide the question by means of the present results, the latter alternative has been shown to present an attractive explanation.

The present results have been compared with the more important kinetic investigations and current theories and seem to be in agreement with most of them in so far as the former have a bearing on the latter.

Nobel Institute of Physics, Stockholm 50.

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Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 13

Communicated 25 January 1950 by JOHN RUNNSTRÖM

On protein fractions in sea urchin eggs and their changes upon fertilization

By SVEN LINDVALL and ASTRID CARSJÖ

With 2 figures in the text

According to a theory emphazised particularly by HEILBRUNN (2) activation means gelation of the egg protoplasm. He found that a very marked increase in viscosity follows fertilization, and suggested that this gelation is similar to that of blood clotting. RUNNSTRÖM (5) showed that the gel forming elements of the cytoplasm are aggregated in the form of loosely connected clusters. Upon fertilization the gel forming elements are dispersed into a three dimensional hydrated more rigid framework.

These investigations show that thorough changes occur at fertilization with respect to the physico-chemical state of the egg proteins. In a more quantitative way this was demonstrated by MIRSKY (3) who extracted lyophilized eggs with 1 N KCl-solution. He then found that the extractable proteins decreased with 12 per cent at the fertilization. With the aid of electrophoretic experiments MONROY (4) also found changes in the proteins from water extracts of lyophilized eggs. According to this author a new component arises immediately upon fertilization, but disappears about 30 minutes later.

In order to study these conditions more closely, attempts were made to fractionate the proteins in eggs previously deprived of their jelly coats with the aid of the method introduced by DERRIEN (1). The successive precipitations brought about by addition of $AmSO_4$ are filtered, and the extinctions of the clear filtrate solutions are evaluated at 2 700 Å. The extinction values are recorded in a diagram and a staircase-like curve is obtained. Each step refers to one protein fraction.

The material which consists of lyophilized unfertilized or fertilized eggs of *Strongylocentrotus droebachiensis*, is extracted under dialysis against a 1 N KCl-solution. With the aid of a phosphate buffert the pH of this solution had been adjusted to 7.25. The results obtained have borne out MIRSKY's results regarding the difference in soluble N between unfertilized and fertilized eggs. The material was ground in a mortar in order to disintegrate the eggs. The result was satisfactory regarding the unfertilized eggs, but despite the grinding the fertilized eggs seemed rather intact when examined under a microscope. The objection could thus be raised that the coarser state of the ground fertilized eggs prevented the complete extraction of N-containing substances soluble in 1 N KCl-solution. A satisfactory disintegration of both unfertilized and fertilized eggs was brought about by using a vibrator in which the eggs suspended in KCl-solution were ground with glass pearls. The yield of sol-

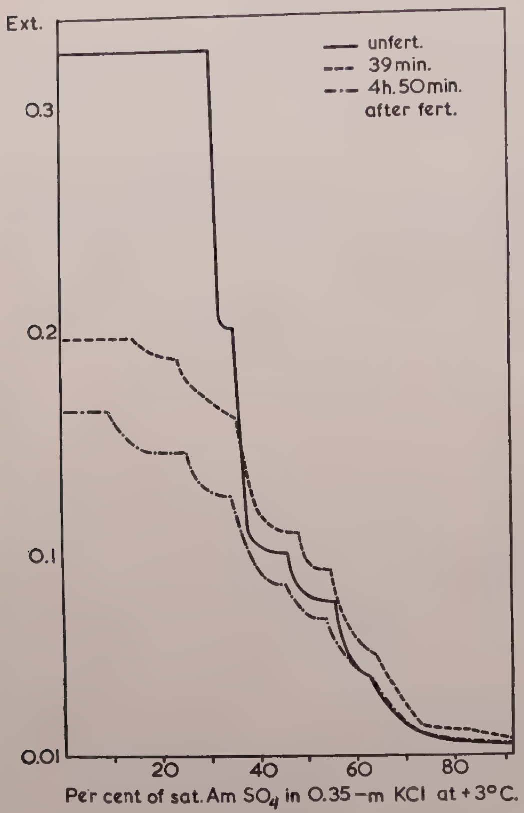

Fig. 1.

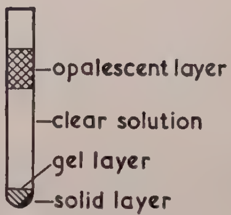

Fig. 2.

Fig. 1. Curves indicating the different protein fraction obtained in 1 N KCl extracts from unfertilized and fertilized sea urchin eggs according to DERRIEN's (1) method. Fig. 2. Diagrammatic representation of the different layers formed upon centrifuging a 1 N KCl extract of unfertilized sea urchin eggs at 65 000 × g.

uble N was, however, not increased. After having been dialyzed against KCl, the suspension was centrifuged for one hour at 8 000 × g. The somewhat opalescent solution then obtained was fractionated according to the method indicated above. The difference between unfertilized and fertilized eggs resides especially in the protein fractions that precipitate at an $AmSO_4$-concentration lower than 40 per cent (Fig. 1).

In order to remove the opalescence the extracts were centrifuged at 65 000 × g for two hours. A solid layer was then formed at the bottom of the tube both in extracts from unfertilized and from fertilized eggs (Fig. 2). Moreover, an opalescent layer had separated out at the top of the solution. This layer probably corresponds to a lipid material which along with the heavier solid material gives rise to the opalescence. It was of particular interest that in extracts from unfertilized eggs a semiliquid gel layer separated out above the solid layer. The gel layer did not appear in extracts from fertilized eggs. This layer seems to correspond to the fraction which precipitates at an Am SO_4-concentration lower than 40 per cent or at least to a part of this fraction. After fertilization the semiliquid gel which flows on tilting the tube must be transformed into solid material.

Eggs from the material available at present have also been extracted with distilled water.[1] The differences in soluble N between extracts from unfertilized and fertilized eggs were shown to occur also after water extraction. When adding increasing concentration of Ca to these extracts, a maximum precipitation was obtained at appr. 0.02–0.03 M $CaCl_2$ in extracts of unfertilized eggs, whereas no precipitation with Ca occurred in extracts from fertilized eggs.

This work was carried out as part of a research program organized by Professor J. Runnström with the support of the Swedish Natural Science Research Council.

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[1] This material has been stored for almost one year in lyophilized state. In view of the long storage time secondary changes cannot be excluded with certainty.

Tryckt den 19 maj 1950

Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 14

Vorgelegt am 22. Februar 1950

Neue Redukton-Derivate

Von Hans von Euler und Hans Hasselquist

Teilnahme des Triose-Reduktons an biologischen Vorgängen

Triose-Redukton, das Derivat einfacher Zuckerarten, beteiligt sich an Synthesen in der lebenden Zelle — es sei nur an die Bildung von Pterinen aus Aminopyrimidinen und Redukton erinnert[1] — und kann also die Teilnahme dieser Zuckerarten an biologischen Vorgängen vermitteln. O'Meara[2] hat darauf hingewiesen, dass das in Bakterienkulturen entstehende Redukton in Verbindung mit p-Aminobenzoesäure als Wuchsstoff gewisser Bakterien fungieren kann, und weiterhin, dass das Redukton in der Zelle durch p-Aminobenzoesäure stabilisiert wird. Hierauf, wie auf die Hypothese des gleichen Forschers, dass die Sulfonamide, indem sie das Redukton im biologischen Medium binden, seine anderweitige Ausnützung verhindern, wollen wir hier nicht näher eingehen.

Kondensationsprodukte von Triose-Redukton mit p-Aminobenzoesäure, Sulfanilamiden, Sulfapyridin und Sulfathiazol wurden von O'Meara und seinen Mitarbeitern dargestellt. Ausgehend von dieser Arbeit haben dann H. S. Forrest und James Walker[3] solche Kondensationsprodukte näher untersucht, nachdem schon Angier und Mitarb.[4] p-Aminobenzoesäure und Redukton kondensiert hatten. Analoge Produkte wurden auch in diesem Institut hergestellt und beschrieben.[5]

p-Aminobenzoesäure und Triose-Redukton

Wie wir in einer vorhergehenden Mitteilung (Rec. 69, 1950) kurz erwähnten, erhielten wir durch Einwirkung von Redukton auf p-Aminobenzoesäure, in Bestätigung der Angaben von Angier et al. (Am. Cyanamid Comp.) (l.c.) das Produkt $C_{10}H_9O_4N$, ein gelbes Pulver für das wir den Zersetz.pt. 264° fanden; demselben kommt nach Analyse und Eigenschaften unzweifelhaft das Komponenten-Verhältnis 1 : 1 und somit die Formel (I) zu:

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[1] Siehe hierzu Nimmo-Smith, Lascelles u. Woods, Brit. J. exp. Path. 29, 264 (1948).

[2] R. A. Q. O'Meara, P. A. McNally u. H. G. Nelson, Nature, 154, 796 (1944). — Lancet, II, 747 (1947). — E. A. Bell, W. Cocker a. R. A. Q. O'Meara, Biochem. J. 45, 373 (1949).

[3] H. S. Forrest u. James Walker, Soc. 1949, 97. — Nature, 161, 308 (1948).

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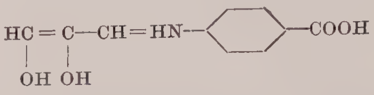
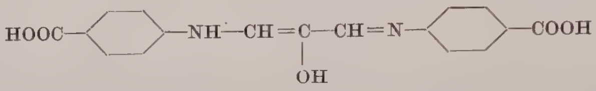
[5] Euler, H. Hasselquist u. U. Lööv, Dieses Arkiv 1, Nr. 60 (1950). — Euler u. H. Hasselquist, Rec. Trav. Chim. Pays-Bas 69, 402 (1950).



Löslich in viel Pyridin. Unlöslich in allen gewöhnlichen Lösungsmitteln.

Die gleiche Substanz erhielten wir auch durch Anwendung der Reaktionskomponenten in anderen Mengen-Verhältnissen als 1 : 1. Für den abweichenden Befund von Forrest und Walker (l.c., S. 297), welche angeben, ein Produkt vom Komponenten-Verhältnis 2 : 1, entsprechend der Formel (II)



erhalten zu haben, können wir keine Erklärung geben.

Kondensation des p-Aminobenzoesäure-β-chloräthylesters mit Redukton

Das Redukton-Derivat der p-Aminobenzoesäure ist, wie erwähnt, in Wasser fast unlöslich, wodurch seine Anwendung zu biochemischen Arbeiten erschwert wird. Nach unseren früheren Untersuchungen über Cholinester der p-Aminobenzoesäure[1] war vorauszusehen, dass die Löslichkeitsverhältnisse sich günstiger gestalten, wenn die Cholingruppe in das Reduktonderivat der p-Aminobenzoesäure eingeführt wird. Bei der Darstellung des Redukton-p-Aminosäure-Cholinesters konnten wir uns auf unsere früheren an Cholinestern gewonnenen Erfahrungen stützen.

Es wurde also zunächst p-Aminobenzoesäure-β-chloräthylester aus p-Aminobenzoesäure und Äthylen-chlorhydrin nach der Vorschrift des DRP 194748 dargestellt.[2] Der p-Aminobenzoesäure-β-chloräthylester wurde zunächst als Ausgangsmaterial für das entsprechende Redukton-Derivat verwendet.

1,99 g Ester wurden in 30 ml Wasser unter Zusatz von einigen ml Aceton aufgeschlämmt, worauf die Lösung mit 0,88 g Redukton auf dem Wasserbad erwärmt wurde bis gelbe Flocken ausfielen. Nach Abkühlung des Reaktionsgemisches wurden die Flocken abfiltriert und mit Wasser und Äther gewaschen. Ausbeute 1,92 g Rohprodukt, Schmpt. 185°–188°. Durch Umkrystallisieren aus Aceton stieg der Schmpt. auf 187–188°, und durch weiteres Umkrystallisieren aus 96 %igem Äthanol wurden gelbe, glänzende Blättchen erhalten, die bei 188,5° unter Färbung aber ohne sichtbare Zersetzung schmolzen (Produkt HH 99).

Subst. HH 99 ist bei Zimmertemperatur in Wasser und allen gewöhnlichen Lösungsmitteln schwer löslich, löst sich aber in der Wärme leicht in Aceton, Äthanol, Äthylacetat, Eisessig und Chloroform, nur wenig in Benzol und gar nicht in Petroleumäther.

Die Substanz gibt mit $FeCl_3$ in alkoholischer Lösung sofort eine Blaufärbung, die bald in stark violett übergeht und allmählich verschwindet.

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[1] H. v. Euler, U. S. v. Euler u. H. Hasselquist, Arkiv f. Kemi etc. 20 A, Nr. 16 (1945).

[2] Siehe auch Euler u. B. Högberg, Arkiv f. Kemi etc. 19 B, Nr. 10 (1945).

Titration mit Tillmans Reagenz (TR).

10,6 mg HH 99 in 20 ml Äthanol gelöst wurde in Stickstoff-Atmosphäre mit einer 0,01 n. TR-Lösung unter gelinder Erwärmung (ca. 30°) titriert. Verbrauch 7,7 ml TR-Lösung.

$$E_{\text{gef}} = \frac{10{,}6}{7{,}7 \cdot 0{,}01} = 138$$

$$E_{\text{ber}} \text{ für } C_{12}H_{12}O_2NCl = 135$$

Es wurde also auf 1 Mol Sbst. HH 99 sehr angenähert 1 Mol TR verbraucht.

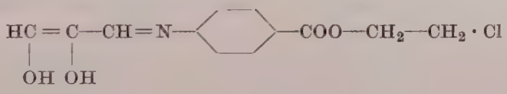
Chlor-Bestimmung.

58,7 mg HH 99 + 10 ml Wasser + Überschuss von Silbernitrat-Lösung wurden mit 10 ml konc. Salpetersäure 30 Minuten zum Sieden erhitzt. Dann wurde die Lösung mit 100 ml Wasser verdünnt und aufgekocht. Das gebildete AgCl wurde abfiltriert, mit Eisessig, Äthanol und Äther gewaschen, getrocknet und gewogen. Gebildetes AgCl = 29,2 mg.

$$Cl_{\text{gef}} = \frac{29{,}2 \cdot 0{,}2474}{58{,}7} = 12{,}3\ \%\ Cl$$

$$Cl_{\text{ber}} \text{ für } C_{12}H_{12}O_2NCl = 13{,}2\ \%\ Cl$$

Die gelbe Farbe der Substanz 99, wie anderer Redukton-p-Aminobenzoesäure-Verbindungen, dürfte durch die konjugierten Doppelbindungen hervorgerufen sein.



Kondensation von p-Aminobenzoesäure-cholinchlorid mit Redukton

p-Aminobenzoesäure-β-chloräthylester wurde 24 Stunden bei 60° mit Trimethylamin in verschlossenem Rohr erhitzt. Das wiederholt umkrystallisierte Produkt $C_{12}H_{19}O_2N_2Cl + H_2O$ zeigte den richtigen Schmpt. 216,5°.

0,54 g p-Aminobenzoyl-cholinchlorid, in 20 ml abs. Alkohol gelöst, wurden mit einer Lösung von 0,185 g Redukton in 5 ml abs. Alkohol versetzt. Es trat sofort deutliche Gelbfärbung ein und nach einiger Zeit fielen gelbe, verfilzte Nadeln aus, die in Lösung stark mit grüner Farbe fluorescierten. Die Menge der abfiltrierten und gewaschenen Krystalle betrug 430 mg. Dieses Rohprodukt schmolz unter Braunfärbung und Zersetzung bei 248°. Durch 2-maliges Umkrystallisieren aus Methanol-Aceton änderte sich der Schmpt., 248°, nicht.

Die Sbst. HH 100 löst sich in Wasser sehr leicht mit gelber Farbe; sie ist ferner löslich in warmem Äthanol und Methanol; wenig löslich in Äther, Aceton, Chloroform und Eisessig.

Redukton-Derivate der 4-Amino-salicylsäure

Umsetzung der 4-Amino-salicylsäure mit Äthylenchlorhydrin

5 g 4-Amino-salicylsäure wurden mit 15 ml Äthylenchlorhydrin + 3 ml konc. Schwefelsäure 15 Minuten mit Rückflusskühler gekocht. Die abgekühlte Lösung wurde mit dem doppelten Volumen Wasser verdünnt und mit Soda neutralisiert wodurch kleine, braunglänzende Prismen ausfielen. Die Ausbeute an Rohprodukt, vom Schmpt. 116–117,5° betrug 3,2 g. Durch zweimaliges Umkrystallisieren aus Benzol der immer noch braunen Krystalle stieg der Schmpt. auf 119–120°. Nach weiterer Umkrystallisation aus Äthanol wurden grosse, beinahe farblose Prismen erhalten, der Schmpt. derselben betrug aber nur 118,5–119°.

Die Sbst. HH 101 ist leicht löslich in Methanol, Äthanol, Äther, Äthylacetat und Chloroform, löslich in Benzol, aber wenig löslich in Wasser und in Petroleumäther.

$FeCl_3$ in alkoholischer Lösung gibt mit HH 101 eine violettrote Farbe.

Chlor-Bestimmung.

50,2 mg Sbst. wurden in Alkali 15 Minuten geschmolzen und nach Abkühlen der Masse mit Salpetersäure angesäuert. Die Fällung mit Silbernitratlösung ergab 33,2 mg AgCl.

$Cl_{gef} = 16{,}36\ \%\ Cl$ $\qquad N_{gef} = 6{,}35$

Cl_{ber} für $C_9H_{10}O_3NCl = 16{,}44\ \%\ Cl$ $\qquad N_{ber} = 6{,}50$

Umsetzung von 4-Amino-salicylsäure-β-chloräthylester mit Trimethylamin

1,5 g Ester wurden mit 15 ml einer benzolischen Lösung von 1,5 g Trimethylamin bei 100° im Bombenrohr 18 Stunden lang erhitzt; dabei schieden sich Krystallblätter ab. Nach Abkühlen des Röhreninhaltes wurden die Krystalle abfiltriert und gewaschen. Das Rohprodukt, 800 mg, enthielt noch etwas Ausgangsmaterial, schmolz bei 160–175°. Die Krystalle wurden mit Aceton aufgekocht, worin das Reaktionsprodukt sich nicht löst; der Schmpt. stieg nun auf 201–202° (Zersetzung), und nach weiterem Umkrystallisieren aus wenig 96 %igem Äthanol auf 207° (Zersetzung).

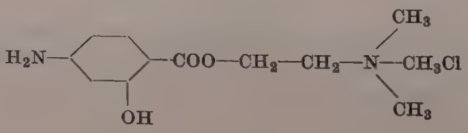
Die Sbst. HH 102 bildet schwach braun gefärbte tafelförmige Krystalle leicht löslich in Wasser und Alkohol, wenig löslich in Benzol, Aceton, Äther und Äthylacetat. Mit $FeCl_3$ liefert die wässrige Lösung eine rote bis rotviolette Farbe. Beim Kochen mit Alkali wird freies Trimethylamin abgespalten.

Chlor-Bestimmung.

27,0 mg Sbst. HH 102 gaben 13,7 mg AgCl.

$Cl_{gef} = 12{,}55\ \%$ $\qquad N_{gef} = 9{,}67$

Cl_{ber} für 4-Amino-salicylcholinchlorid, $C_{12}H_{19}O_3N_2Cl = 12{,}91\ \%$ $\qquad N_{ber} = 10{,}20$



Umsetzung von 4-Amino-salicylsäure-β-chloräthylester mit Redukton

88 mg Redukton in 20 ml Wasser wurden mit 216 mg Chloräthylester + einigen ml Aceton auf dem Wasserbad erwärmt bis Flocken von orange-gelber Farbe ausfielen. Dieselben wurden abfiltriert und mit etwas Äther gewaschen. Das so erhaltene Rohprodukt, 221 mg (77 % der Theorie) schmolz bei 183–185°. Nach einer Umkrystallisation aus Alkohol stieg der Schmelzpunkt auf 194–195°, nach weiteren 2 Umkrystallisationen aus Äthylacetat-Petroleumäther wurde der Schmpt. bei 197,5° konstant (Dunkelfärbung trat bei 196° ein).

Die Sbst. HH 103 bildete orangegelbe, silberglänzende Blättchen. Dieselben sind leichtlöslich in warmem Alkohol, Äthylacetat und Aceton, etwas löslich in Äther und Benzol, schwerlöslich in Wasser und Petroleumäther.

HH 103 enthält eine Endiol-Gruppe, wie die Reduktion von TR zeigt:

$$E_{\text{gef}} = \frac{6{,}9}{4{,}8 \cdot 0{,}01} = 144$$

$$E_{\text{ber}} \text{ für } C_{12}H_{12}O_5NCl = 143$$

Chlor-Bestimmung.

33,6 mg Sbst. wurde nach der Alkalischmelze in Wasser gelöst, mit Salpetersäure angesäuert und mit Silbernitrat gefällt. Erhalten wurden 16,1 mg.

$$Cl_{\text{gef}} = \frac{16{,}1 \cdot 0{,}02476}{33{,}6} = 11{,}87\ \%$$

$$Cl_{\text{ber}} = \ldots\ldots\ldots\ldots\ldots\ 12{,}41\ \%$$

Umsetzung von 4-Amino-salicylsäure-cholinchlorid mit Redukton

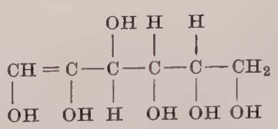
550 mg 4-Amino-salicylsäure-cholinchlorid wurde mit einer kalten Lösung von 200 mg Redukton in 2 ml abs. Alkohol versetzt. Während 1 Stunde färbte sich das Reaktionsgemisch bei Zimmertemperatur stark gelb und kugelförmige Krystalle fielen aus. Nach Ablauf der Reaktionszeit wurden 3 ml abs. Alkohol zugesetzt, worauf die Krystalle abfiltriert und mit Alkohol und Äther gewaschen wurden. Das Rohprodukt, 200 mg, schmolz unter Zersetzung bei 195–205°.

Die Sbst. HH 104 löst sich äusserst leicht in Wasser mit intensiv gelber Farbe. Schwerlöslich in allen gewöhnlichen organischen Lösungsmitteln. Gibt mit $FeCl_3$ in wässriger Lösung eine schnell verschwindende blau-grüne Färbung.

In fein verteilter Form fluoresciert die Sbst. HH 104 stark grün. Eine wässrige Lösung derselben zeigt im ultravioletten Licht gelbgrüne Fluorescens, welche (wie auch Sbst. HH 100) auf Zusatz von Alkali stark abnimmt aber nicht ganz verschwindet.

Zur Kenntnis der Enolisierung in der Zuckergruppe

Wie schon vor langer Zeit von LOBRY DE BRUYN und A. VAN EKENSTEIN[1] beobachtet worden ist, erfahren Aldo- und Keto-Hexosen in alkalischer Lösung eine Umlagerung, welche zu einem Gleichgewicht zwischen Glukose, Fruktose und Mannose führt. Unter den Endiol- bezw. Endiolatformen, welche dabei als Zwischenprodukte in Betracht kommen, haben wir zunächst — wie KARRER — der Formel



$$\begin{array}{ccccccccccc} & & & & \mathrm{OH} & & \mathrm{H} & & \mathrm{H} & & \\ & & & & | & & | & & | & & \\ \mathrm{CH} & = & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{C} & - & \mathrm{CH_2} \\ | & & | & & | & & | & & | & & | \\ \mathrm{OH} & & \mathrm{OH} & & \mathrm{H} & & \mathrm{OH} & & \mathrm{OH} & & \mathrm{OH} \end{array}$$

den Vorzug gegeben.[2]

Die Isolierung dieses oder isomerer Hexose-Endiolate ist bisher nicht gelungen, obwohl nach unseren Versuchen sich das erwähnte LOBRY DE BRUYN'sche Gleichgewicht schneller einstellt als der Bildung des Reduktons bezw. der TR-Entfärbung entspricht.[3] Immerhin ist die Endiolat-Bildung aus z. B. Glukose keine augenblicklich verlaufende Reaktion. Triose-Redukton entsteht, wie wir früher gezeigt haben, durch alkalische Behandlung der Glukose, ohne Mitwirkung eines Oxydationsmittels, nur zu etwa 5 %. In Gegenwart von TR wird auf 1 Mol Glukose angenähert 1 Mol Endiolat gebildet.

Hexoson hat EMIL FISCHER bekanntlich durch Spaltung von Phenylglukosazon mit starker Salzsäure gewonnen und hat das Produkt, das er nicht krystallisiert erhalten und dessen physikalische Konstanten er nicht bestimmen konnte, in folgender Weise formuliert:

$$\mathrm{CHO{-}CO{-}CH(OH){-}CH(OH){-}CH(OH){-}CH_2(OH)}$$

Obwohl EMIL FISCHER sehr selten einen von ihm dargestellten principiell wichtigen Stoff verlassen hat ohne ihn vollständig aufzuklären, ist er auf die Reinigung seines Osons später nicht mehr zurückgekommen.

Stellt man Glukoson — wie bekannt identisch mit Fruktoson und Mannoson — aus dem Phenylosazon dar und zwar indem man die beiden Phenylhydrazinreste nicht durch Salzsäure abspaltet sondern mit Benzaldehyd zerlegt[4], so erhält man das Oson als rein weisses, in Wasser leicht lösliches Pulver.

Durch Einwirkung von verdünntem Alkali wirkt die wässrige Osonlösung schon bei Zimmertemperatur stark reduzierend auf den Dichlor-phenol-indophenol-Farbstoff (TR). Nach quantitativen Titrations-Versuchen mit TR, die wir bis jetzt an dem nicht krystallisierten Osonpulver ausführen konnten, scheint sich auf 1 Mol Oson nur $^1/_2$ Endiol-Gruppe zu bilden.

E. FISCHER[5] betonte in seinen beiden Mitteilungen über Oson schon die starke Reduktionswirkung dieses Stoffes gegen Fehlings Lösung und seine Empfindlichkeit gegen Alkalien. Hier muss besonders darauf hingewiesen werden, dass sich diese hohe Reduktionskraft *nicht* in saurer oder neutraler Lösung geltend macht, also in ausgesprochenem Gegensatz zu den Verhältnissen bei Ascorbinsäure und Triose-Redukton. Erst durch die Endiolat-Bildung wird Oson ein Reduktionsmittel für das Tillmans-Reagenz, es übertrifft aber in dieser Hinsicht die alkalisch vorbehandelten Hexosen.

Stockholm 6, Vitamin-Institut.

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[1] LOBRY DE BRUYN u. A. VAN EKENSTEIN, Rec. Trav. Chim. Pays-Bas 16, 257 (1897).

[2] KARRER-Festschrift, Experientia, 5, 81 (1949). — Chimia, 3, 209 (1949). — P. KARRER, Lehrbuch d. org. Chemie, 10 Aufl., S. 353.

[3] EULER u. HASSELQUIST, Dieses Arkiv 1, Nr. 30 (1949). — 26 A, Nr. 25 (1948).

[4] P. MAYER, Biochem. Z. 40, 456 (1912).

[5] E. FISCHER, Ber. 21, 2631 (1888). — 22, 27 (1889).

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A new method for the calculation of reaction velocity constants

By SIGVARD WIDEQVIST

The method described in the present publication has been worked out for two purposes, namely

1) to calculate a second order reaction velocity constant, k_2, without using the initial concentrations of the reacting substances,

2) to calculate the first and second order velocity constants, k_1 and k_2, for a simultaneous first and second order reaction from *one single run* of measurements.

The method which involves the use of graphical integration is, as the author has found, not previously described in the chemical literature.

Let us regard the reaction

$$\begin{array}{cccc} \mathrm{A} & + \; \mathrm{B} & \rightarrow \mathrm{C} & + \; \mathrm{D} \\ a-x & b-x & x & x \end{array}$$

For convenience the symbols employed are summarized below:

t_n = time from the start of the reaction (minutes)

t_0 = arbitrary zero time; time for the first sample

$t = t_n - t_0$

a_0 and b_0 = actual initial concentrations of A and B, i.e. in that moment, when the reacting substances are mixed together

$(a_0 - x_0)$ and $(b_0 - x_0)$ = concentrations of A and B at the time t_0

$(a_0 - x_n)$ and $(b_0 - x_n)$ = concentrations of A and B at the time t_n

$x = x_n - x_0$

$a = a_0 - x_0$

$b = b_0 - x_0$

k_1 = first order reaction velocity constant

k_2 = second order reaction velocity constant

$c = k_1/k_2 + a$

A = area between the curve $(b - x) = F(t)$ and the t-axis.

In the general case the reaction velocity may be represented by the differential equation

$$d\,x/d\,t = k_1\,(b_0 - x_n) + k_2\,(b_0 - x_n)\,(a_0 - x_n). \tag{1}$$

On putting $k_2\,(b_0 - x_n)$ outside the brackets we get

$$d\,x/d\,t = k_2\,(b_0 - x_n)\,(k_1/k_2 + a_0 - x_n) \tag{1 a}$$

or, if we put $k_1/k_2 + a_0 = c_0$

$$d\,x/d\,t = k_2\,(b_0 - x_n)\,(c_0 - x_n). \tag{1 b}$$

On integration we obtain

$$k_1 + k_2\,(a_0 - b_0) = \frac{1}{0.4343\,t_n}\log\frac{b_0\,[k_1 + k_2\,(a_0 - x_n)]}{(b_0 - x_n)\,(k_1 + k_2\,a_0)} \tag{2}$$

resp.

$$k_2 = \frac{1}{0.4343\,(k_1/k_2 + a_0 - b_0)\,t_n}\log\frac{b_0\,(k_1/k_2 + a_0 - x_n)}{(b_0 - x_n)\,(k_1/k_2 + a_0)} \tag{2 a}$$

and

$$k_2 = \frac{1}{0.4343\,(c_0 - b_0)\,t_n}\log\frac{b_0\,(c_0 - x_n)}{c_0\,(b_0 - x_n)}. \tag{2 b}$$

During the reaction the concentration $(b_0 - x_n)$ is determined. The first sample is taken at an arbitrary zero time t_0. Thus we have

$$k_2 = \frac{1}{0.4343\,(c_0 - b_0)\,t_0}\log\frac{b_0\,(c_0 - x_0)}{c_0\,(b_0 - x_0)}. \tag{3}$$

Combining equ. (2 b) and (3) we get

$$k_2 = \frac{1}{0.4343\,(c_0 - b_0)\,(t_n - t_0)}\log\frac{(b_0 - x_0)\,(c_0 - x_n)}{(c_0 - x_0)\,(b_0 - x_n)}.$$

Put $(c_0 - x_0) = c$, $(b_0 - x_0) = b$, $(a_0 - x_0) = a$, $(x_n - x_0) = x$ and $(t_n - t_0) = t$. Then we get

$$c_0 - b_0 = (c_0 - x_0) - (b_0 - x_0) = c - b$$

$$c = k_1/k_2 + a$$

and

$$k_2 = \frac{1}{0.4343\,(c - b)\,t}\log\frac{b\,(c - x)}{c\,(b - x)}. \tag{4}$$

From a run of $(b - x)$-values we may calculate k_2, when $(c - b)$ is known. That is the case, when a_0 and b_0 are known and $k_1 = 0$. Then $c = a$, and we have the ordinary second order reaction, where

$$k_2 = \frac{1}{0.4343\,(a_0 - b_0)\,t} \log \frac{b\,(a - x)}{a\,(b - x)}. \tag{5}$$

When $k_2 = 0$, we have the first order reaction and get

$$k_1 = \frac{1}{0.4343\,t} \log \frac{b}{b - x}. \tag{6}$$

When k_1 and $k_2 \neq 0$, we must know both $(a - b)$ and k_1/k_2, when k_2 is to be calculated from equ. (4).

Now we shall demonstrate, how it is possible to calculate k_2 without using any quantity except a run of $(b - x)$-values. When the difference $(a - b)$ is known, we may also calculate k_1 in the case of a mixed first and second order reaction.

Equ. (4) may also be written

$$0.4343\,k_2\,(c - b)\,t = \log b/(b - x) - \log c/(c - x). \tag{4 b}$$

Now we shall eliminate $\log c/(c - x)$. In order to do that we introduce the variable A, defined as follows

$$A = \int_0^t (b - x)\,d\,t. \tag{7}$$

A is easily determined. By plotting $(b - x)$ against t we find that A is the area between the curve and the t-axis. The area may be determined by weighing or by other methods.

According to equ. (1 b) we have

$$(b - x)\,d\,t = \frac{d\,x}{k_2\,(c - x)}.$$

On introduction of this expression into equ. (7) we get

$$A = \frac{1}{k_2} \int_0^x \frac{d\,x}{c - x} = \frac{1}{k_2}\, ln\, \frac{c}{c - x} = \frac{1}{0.4343\,k_2} \log \frac{c}{c - x}. \tag{8}$$

Thus

$$\log c/(c - x) = 0.4343\,k_2\,A.$$

When this value is introduced into equ. (4 b) we obtain

$$A = \frac{1}{0.4343\,k_2} \log \frac{b}{b - x} - (c - b)\,t. \tag{9}$$

By means of equ. (9) both k_2 and the difference $(c - b)$ may be calculated from a run of corresponding $(b - x)$- and t-values.

Further we have

$$c = k_1/k_2 + a.$$

Thus

$$c - b = k_1/k_2 + a - b.$$

But

$$a - b = a_0 - b_0 .$$

Thus

$$c - b = k_1/k_2 + (a_0 - b_0)$$

and

$$k_1 = k_2 [(c - b) - (a_0 - b_0)]. \qquad (10)$$

Thus, when the initial concentrations a_0 and b_0 are known, we may calculate both k_1 and k_2 from equ. (9) and (10).

The calculation of k_2 and $(c - b)$ is conveniently performed by means of the least squares method. Let us put $\log b/(b - x) = z$, $(c - b) = s$ and $1/0.4343\, k_2 = r$. Then we get

$$A = r z - s t. \qquad (11)$$

A is now a function of the known variables z and t. Let us regard the function

$$F = \sum (A - r z + s t)^2. \qquad (12)$$

On partial derivation with respect to the parameters we get

$$\partial F/\partial r = -2 [\sum A z - r \sum z^2 + s \sum z t] \qquad (13)$$

$$\partial F/\partial s = \quad 2 [\sum A t - r \sum z t + s \sum t^2]. \qquad (14)$$

F has minimum, when $\partial F/\partial r$ and $\partial F/\partial s = 0$. Thus we obtain the equation system

$$\left.\begin{aligned} \sum A z - r \sum z^2 + s \sum z t &= 0 \\ \sum A t - r \sum z t + s \sum t^2 &= 0 \end{aligned}\right\} \qquad (15)$$

On resolution of the parameters we obtain

$$r = \frac{\sum A z \sum t^2 - \sum A t \sum z t}{\sum z^2 \sum t^2 - (\sum z t)^2} \quad \text{and} \quad s = \frac{\sum A z \sum z t - \sum A t \sum z^2}{\sum z^2 \sum t^2 - (\sum z t)^2}$$

and on putting $1/r = 0.4343\, k_2$ and $s = (c - b)$

$$0.4343\, k_2 = \frac{\sum z^2 \sum t^2 - (\sum z t)^2}{\sum A z \sum t^2 - \sum A t \sum z t} \qquad (16)$$

$$c - b = \frac{\sum A z \sum z t - \sum A t \sum z^2}{\sum z^2 \sum t^2 - (\sum z t)^2}. \qquad (17)$$

Examples

I. The simple second order reaction

The saponification of ethyl acetate

The data of TERRY and STIEGLITZ (1) for the bimolecular hydrolysis of ethyl acetate by sodium hydroxide have been treated by the method above. The initial concentrations of sodium hydroxide and ethyl acetate were resp. 0.010010 and 0.008072. The concentration of sodium hydroxide was determined during the reaction. The experimental data of TERRY and STIEGLITZ are reproduced in Table I. The velocity constant k_2 was calculated in the usual way according to equ. (5).

We assume that the concentration of ethyl acetate $(a_0 - x_n)$ is unknown. The time for the first sample, $t_n = 2.27$, is taken as the zero time, t_0. Then

Table I

The saponification of ethyl acetate by sodium hydroxide. $a_0 = 0.008072$, $b_0 = 0.10010$, $t_0 = 2.27$; 24.92° C. (TERRY and STIEGLITZ (1)).

t_n	$b_0 - x_n$	k_2
2.27	0.008934	—
3.01	8630	—
5.85	7669	6.71
6.83	7392	6.72
8.02	7085	6.72
8.92	6872	6.70
10.05	6632	6.68
10.96	6441	6.71
11.93	6258	6.71

Mean value 6.71

Table II

The saponification of ethyl acetate.

t	z	A
0.74	0.01504	0.00650
3.58	.06631	2965
4.56	.08229	3703
5.75	.10071	4564
6.65	.11397	5192
7.78	.12971	5955
8.69	.14210	6550
9.66	.15461	7166

we obtain the values in Table II for A and z. As the reaction is of the second order, $k_1 = 0$ and $(c - b) = (a - b) = (a_0 - b_0)$. On applying equ. (16) and (17) to the data of Table II we obtain

$$k_2 = 6.78 \text{ and } (b_0 - a_0) = 0.001945.$$

In good agreement with this, TERRY and STIEGLITZ reported $k_2 = 6.71$ and $(b_0 - a_0) = 0.001938$.

II. The simultaneous first and second order reaction

1. The hydrolysis of sodium α-bromopropionate by sodium hydroxide

The reaction

$$\underset{b - x}{CH_3\text{—}CHBr\text{—}COO^-} + \underset{a - x}{OH^-} \rightarrow \underset{x}{CH_3\text{—}CH(OH)\text{—}COO^-} + \underset{x}{Br^-}$$

follows the differential equation

$$dx/dt = k_1(b - x) + k_2(b - x)(a - x).$$

This reaction has been investigated by COWDREY, HUGHES and INGOLD (2) CHADWICK and PACSU (3), GRUNWALD and WINSTEIN (4) and others. These authors have tried to determine both k_1 and k_2. It is, however, doubtful whether their velocity constants are fairly correct.

Table III

The hydrolysis of α-bromopropionic acid by alkali. $a_0 = 0.1080$, $b_0 = 0.0931$, $t_0 = 0$; 64° C. (COWDREY, HUGHES and INGOLD (2)).

t	$b - x$	$k_1 \cdot 10^3$
0.0	0.0931	
10.8	846	8.85
20	779	8.89
29	722	8.75
40	657	8.70
49	612	8.55
61	558	8.40
77	485	8.48
96	420	8.28
142	300	7.98
171	239	7.95
206	188	7.78
246	141	7.69
299	100	7.46
360	067	7.30

Table IV

Hydrolysis of α-bromopropionic acid.

t	$b - x$	A	z
0	0.0931		
10	852	0.892	0.03825
20	779	1.707	.07725
30	715	2.454	.11491
40	657	3.140	.15111
50	607	3.772	.18587
60	561	4.356	.21973
70	516	4.895	.25645
80	475	5.390	.29238
90	441	5.848	.32503
100	410	6.273	.35656

From the paper of COWDREY, HUGHES and INGOLD the data in Table III are reproduced. The initial concentrations were $a_0 = 0.1080$ and $b_0 = 0.0931$. The rate constant k_1 was calculated by COWDREY, HUGHES and INGOLD according to equ. (5); k_1 is, however, not constant but falls rapidly.

Now we shall calculate the correct values of k_1 and k_2. From Table III we obtain the values in Table IV by plotting $(b - x)$ against t. In order to facilitate the mathematical operations, the $(b - x)$-values are interpolated for tens of minutes. From A and z in Table IV we obtain according to equ. (16) and (17)

$$k_2 = 0.0295$$

and

$$c - b = 0.2165.$$

When these values are introduced into equ. (10) together with a_0 and b_0 we get

$$k_1 = 0.00594.$$

From a number of different series of measurements COWDREY, HUGHES and INGOLD obtained

$$k_1 = 0.00708 - 0.00780$$

and

$$k_2 = 0.0381 - 0.0402.$$

We are now to examine whether the values of k_1 and k_2, obtained by us, may be acceptable. If that is the case, the right hand member in equ. (2) shall be constant. Put this quantity $= K/0.4343$. As is seen from Table V, K is a perfect constant. On division with $0.4343\,(c - b)$, the mean value of K shall give the second order constant k_2. But $(c - b) = 0.2165$. Thus we obtain $k_2 = 0.0295$ which is exactly the same value that was obtained from equ. (16).

Table V

Hydrolysis of α-bromopropionic acid.

t	$K \cdot 10^3$	t	$K \cdot 10^3$
10.8	2.731	96	2.785
20	2.770	142	2.768
29	2.759	171	2.809
40	2.772	206	2.799
49	2.749	246	2.817
61	2.733	299	2.785
77	2.804	360	2.775

Mean value 0.002775

2. The reaction between cinnamyl chloride and potassium acetate in glacial acetic acid

The reaction between cinnamyl chloride and potassium acetate in glacial acetic acid which has been studied by MEISSENHEIMER and BEUTTER (5) proceeds in two ways, namely

$$C_6H_5—CH=CH—CH_2Cl + HOCOCH_3 \rightarrow C_6H_5—CH=CH—CH_2OCOCH_3 + HCl$$

first order

and

$$C_6H_5—CH=CH—CH_2Cl + KOCOCH_3 \rightarrow C_6H_5—CH=CH—CH_2OCOCH_3 + KCl$$

second order

When the concentration of potassium acetate is $(a - x)$ and that of cinnamyl chloride is $(b - x)$, we get the equation

$$dx/dt = k_1 (b - x) + k_2 (a - x) (b - x).$$

According to the measurements of MEISSENHEIMER and BEUTTER we get the data in Table VI; t = time in hours, $t_0 = 0$. The initial concentrations were the same for both substances, namely 0.1000.

From the values in Table VI we get according to equ. (16) and (17)

$$k_2 = 0.1749$$

$$(c - b) = 0.0274.$$

But $a_0 = b_0 = 0.1000$. Thus we get from equ. (10)

$$k_1 = 0.00479.$$

Table VI

The reaction between cinnamyl chloride and potassium acetate. $a_0 = b_0 = 0.1000$, $t_0 = 0$; 50° C. (MEISSENHEIMER and BEUTTER (5)).

t	$a - x$ $b - x$	z	A	K
0	0.1000			
8	.0842	0.07469	0.722	0.002148
24	.0638	.19518	1.918	2083
48	.0448	.34872	3.222	2127
72	.0339	.46980	4.169	2112
96	.0265	.57675	4.878	2116

Mean value 0.002117

$K/0.4343$ is the right hand member of equ. (2). When we put $a_0 = b_0$ in equ. (2), we find that $K = 0.4343\,k_1$. That is also the case, for the mean value of $K = 0.00212$ and $0.4343\,k_1 = 0.00208$.

By means of a cumbersome algebraic analysis of the experimental data MEISSENHEIMER and BEUTTER obtained the values $k_1 = 0.0085$ and $k_2 = 0.13$.

SUMMARY

A new method for the calculation of reaction velocity constants has been described. The method which makes use of graphical integration permits the calculation of second order reaction velocity constants without using the initial concentrations of the reacting substances. In the case of a simultaneous first and second order reaction the rate constants k_1 and k_2 may be calculated, when the initial concentrations are known. The usefulness of the method is illustrated with some examples from the literature.

Department of Organic Chemistry, University of Uppsala.

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Serum fibrinolysin activation in the experimental serum hypersensitiveness of the rabbit

By Hans von Euler and Leo Heller

Attention has been centered many years ago to the high degree of specificity of proteolytic enzymes acting on peptide bonds and on other linkages occurring in normal and pathogenic proteins and protein derivatives. For the peptide splitting enzymes one of the present authors (H. v. E.)[1] has advanced a "dual affinity theory", emphasizing, that ereptic peptidases combine with 2 atomic groupings of the peptide. This hypothesis was later supported by Balls and Köhler[2], and according to J. Fruton[3] modern theories concerning the specificity of proteolytic enzymes are based on this dual-affinity hypotheses. "More recently the finding of synthetic substrates[4] for the protein-splitting enzymes (pepsin, trypsin, chymotrypsin, papain etc.) has led to the extension of this hypothesis to all representative proteolytic enzymes.[5] The relation of the structural and the biological specificities of proteins still constitutes one of the most fascinating problems in protein biochemistry.

The rôle, which proteolysis possibly plays in the mechanism of the anaphylactic reactions, studied by many authors (Rocha e Silva[6] a.o.) has been recently discussed by Ungar and Mist.[7] These authors showed that while the addition in vitro of the specific antigen (egg albumin) to the serum of guinea pigs sensitized against that antigen resulted in the release of active fibrinolysin, the same procedure had no effect on the profibrinolysin present in the serum of normal, unsensitized guinea pigs, which can be otherwise easily activated by a number of different agents and procedures. The activation of the serum protease seemed in that case to have a highly specific character.

If similar phenomena of activated serum proteolysis play a major part in the mechanism of the hypersensitive reactions in general, of the types encountered not only in true anaphylaxis, but also in serum sickness, rheumatic diseases and related conditions, then it should be possible to demonstrate changes in the actual or potential proteolytic activity of the sera of hypersensitive animals or human patients.

Being primarily interested in the mechanism of the *rheumatic diseases* considered to-day by the majority of patologists and clinicians as a manifestation of a peculiar kind of hypersensitivity[8, 9], we wished to study the changes of the serum proteolysis in some kind of experimentally obtainable condition bearing a near relationship to the human rheumatism. The only relatively easily reproducible rheumatism-like condition known to us was the state of hypersensitiveness developing in rabbits treated intravenously with massive doses of horse serum or other antigenically active material, the pathological lesions of which show a striking similarity to those found in the human rheumatic diseases.[10, 11]

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The circumstance that the rabbit, together with many other animal species, does not normally show any noticable fibrinolysin activity[12] when tested by different activation procedures (streptococcal kinase, chloroform extraction etc.) seemed to facilitate the demonstration of eventual appearance of proteolytic activity in the rabbit serum or its euglobulin fraction (supposed to contain the profibrinolysin) concomitantly with the development of hypersensitiveness. The results of some preliminary experiments, performed with the purpose to test the possibility mentioned above, will be reported in the present paper.

Experimental

Materials and methods

Two rabbits (males), weighing respectively 3,500 (rabbit No I) and 4,200 (rabbit No II) gms, were used for the sensitization experiments. Blood samples, 20 ml each, were taken from the ear veins and were allowed to clot. After c:a 6–7 hours at room temperature the serum was centrifuged and sucked off. These sera were used as normal rabbit serum controls in the subsequent fibrinolysin activity determinations. All sera were stored frozen on dry ice.

On the following day both rabbits were injected intravenously with 10 ml per kg body weight of sterile horse serum, heated previously to aproximately 37° C. A transient elevation of the body temperature, measured daily per rectum, corresponding to that described by Rich a. Gregory[10], was observed on the 7th day after the serum injection in the rabbit No II (40.2°), but not in the rabbit No I.

Both rabbits were bled again repeatedly after resp. one week (sera I/II and II/II and three weeks (sera I/III and II/III), and the sera were obtained in the usual way. On the 25th day after the horse serum injection the rabbit No I was injected intravenously into the marginal ear vein with 20 ml of a partially purified streptokinase preparation*, prepared by adsorption on alumina cream (Cathie[13]), and active down to the 1 : 125 dilution when tested against human fibrin clots. The rabbit was bled once more 1 hour after the above injection (serum I/IV).

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* The authors are indebted to Dr. P. E. Hedlund, State Bacteriological Laboratory, Stockholm, for the generous gift of the semipurified streptokinase preparation used throughout our experiments.

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To demonstrate the development and to test the degree of hypersensitivity, skin tests were performed on both hypersensitized rabbits 30 days after the injection of the antigen. Rabbit No III (untreated) was used as a normal control.

0.1 ml volumes of undiluted resp. saline-diluted (1: 10) horse serum were injected intradermally into the shaved back of the animals. The test was read on the following morning, the rabbit No I showing a very strong reaction (with undiluted horse serum — skin necrosis and oedema, with serum diluted 1: 10 — distinct haemorrhage and slight oedema).

The skin response of the other sensitized rabbit was much weaker, the intensity of the reaction against the undiluted serum corresponding approximately to that obtained in the rabbit No I with the serum diluted 1: 10 and the diluted serum giving no reaction at all. The control animal (No III) did not show any reaction either against the undiluted nor against the diluted (1: 10) horse serum.

The animals were than sacrificed, and the organs known to be the predilection site of the characteristic pathological lesions[10, 11] (heart, lung, muscle) were fixed in 10 % formalin for staining and histopathological examination. This should constitute an additional control of the degree to which the characteristic "rheumatoid" lesions of experimentalserum hypersensitiveness have developed following the treatment with horse serum.

At the moment of presenting this preliminary report the results of the microscopical examination of the sections are not yet available. They will be presented later together with the results of further experiments pertaining on the same problem.

Measurements of the proteolytic activity of the serum fibrinolysin were performed with the aid of viscosimetry. The technique originally described by CHRISTENSEN and MACLEOD[14] and used by many other authors (UNGAR[7] a.o.) was used with slight modifications made necessary by the different size of our viscosimeters. Thus a 7 % gelatin solution in phosphate-saline buffer of pH 7,4[7] was substituted for the more dilute solutions used by the authors cited.[7, 14] In our experiments 1 ml of the enzyme solution (see below) mixed with the same volume of phosphate-saline buffer containing the activator were mixed in the viscosimeter with 5 ml of a 7 per cent gelatin solution. Both components were previously heated to the temperature of 37°, and the incubation of the mixture occurred also at that temperature.

The enzyme solution was prepared from freshly thawed serum samples in the form of a serum euglobulin fraction by the technique described in detail by UNGAR[7] and RATNOFF[15] (dilution and isoelectric precipitation at pH 5.2 controlled electrometrically with a glass electrode). The precipitated euglobulin was dissolved in 1/4 of the original volume of phosphate-saline buffer[15], and either used immediately or allowed to age in the refrigerator for a period of a few days.[15] The addition of the activator (see below), with the single exception of the activation by chloroform (see below), occurred immediately before the beginning of the measurements. Flow times were measured 6–7 times for one hour and the changes of viscosity were calculated as by UNGAR and MIST[7] (the plasmin units of CHRISTENSEN[14] were not introduced) for time periods of 30 resp. 60 minutes on the basis of interpolated flow time-incubation time plots. Corrections were made for occasionally occurring spontaneous decreases in the viscosity of the gelatin solutions during incubation. The gelatin blinds were

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Table 1.

Rabbit No	Days interval		Serum No	Aging Time of Euglobulin-solution, (days)	Method of Activation	Viscosity Change: Differences of Flow Times after	
						30 min	60 min
I	0	Bleeding	I (norm)	1	Streptokinase	0	0
	1	10 ml horse serum per kg body wt.					
	9	Bleeding	II	3	»	1.6″	2.6″
	18	»	III	1	»	0	0
				7	»	1.0″	2.0″
				1	Sturin sulph.	0	0
				4	Salmin sulph.	0.1″(!)	0.6″
	24	20 ml Streptokinase interven. — later bleeding	IV	1	Streptokinase	0.1″	0.3″
				(18 hrs)	Chloroform	1.1″	2.4″
				(3 days)	»	0	0
	31	Skin test strongly positive					
II	0	Bleeding	I (norm)	1	Chloroform	0	0
	1	10 ml horse serum per kg body wt.					
	9	Bleeding	II	1	»	0.5″	0.6″
	18	»	III	1	»	0	0
				7	»	0	0
				1	Horse serum (spec. antigen)	0.3″	0.4″
		Control: Horse serum euglobulin alone		(1)	—	(0)	(0)
	31	Skin test weekly positive					
III		Bleeding	I (norm)	1	Streptokinase	0	0
		Skin test negative		(18 hrs)	Chloroform	0.2″	0.6″
		Normal human serum (Control)		1	Streptokinase	1.2″	2.4″
				3	»	2.3″	4.0″
				4	»		
					a) 0.2 ml	1.3″	3.6″
					b) 0.5 ml	1.2″	3.4″

therefore determined immediately following the true determination by substituting phosphate-saline buffer (see above) for the enzyme activator mixture.

The kind of the activation tried in each experiment is shown in Table 1.

When streptococcal kinase from group A strain of hemolytical streptococci was used as activator, 0.5 ml of the activator solution (partly purified by adsorption and elution[13]), was added to 1 ml of the serum euglobulin solution obtained from the respective rabbit serum portion. The above quantity of streptokinase was found to be in considerable excess of that necessary for maximal activation of a sample of normal human euglobulin solution, prepared in exactly the same way as the rabbit serum euglobulin.

In the case of the chloroform activation the technique and the proportions given by RATNOFF[15] were followed in detail. Taking into consideration the relative instability of the chloroform activated enzyme[14, 15], the determinations in that case were carried out c:a 18 hours after the chloroform treatment, the peak of the activity of chloroform-treated serum lying near that time.

The specific antigen used for hypersensitization (horse serum) was also tried for its activating properties, as described by UNGAR and MIST[7] for the sensitized guinea-pig, and their technique was used. Appropriate controls for eventual own proteolytic (fibrinolysin) activity of the horse serum gave negative results, thus simplifying the eventual interpretation of the effectivity of that method of plasmin (fibrinolysin) activation.

In a single experiment, the activation of the rabbit euglobulin solutions by addition of protamin[6] was tried. A heavy precipitate formed on mixing the serum euglobulin solutions with the same volume of salmin resp. sturin solutions in phosphate-buffered saline. After centrifugation the clear supernate (still containing protein material) was tested for the gelatin digesting activity in the usual way.

Results

As mentioned above the aim of our experiments was to demonstrate an eventual increase of the gelatin-digesting activity of the sera of rabbits hypersensitized with horse serum activated in both specific[7] (by the antigen) and unspecific way (by chloroform and by protamines) [14, 15, 6] as well as by bacterial streptokinase. The results of our attempts can not be considered as ultimately conclusive. The majority of the tests performed with different serum portions and different activation methods did not show any proteolytic activity in the gelatin digestion test (Table 1).

Some tentative interpretations of preliminary character seem, however, possible on the basis of the experimental data obtained.

a. Activation by streptokinase

No proteolytic activity could be evoked in the euglobulin preparations obtained from normal (control) rabbit serum by streptokinase doses in excess of those causing maximal activation of human fibrinolysin prepared in an identical way. Also the aging of the normal rabbit euglobulin for 8 days — before the addition of the activator — a method reported to cause gradual spontaneous activation of the proenzyme[15] — remained without any effect.

However, the euglobulin fractions prepared from the portions of the serum obtained from rabbit No I — the animal proven strongly hypersensitive according to the skin tests — gave repeatedly, but not constantly, significantly positive results. The ageing for 3–7 days of the serum euglobulin solutions before addition of the bacterial streptokinase was necessary to demonstrate a gelatin digesting activity of those serum portions, which makes probable the existence of some inhibiting factor in the euglobulin fraction itself, slowly destroyed on standing. We did not, as yet, try whether or not the addition of streptokinase was necessary for demonstrating the activity appearing on standing, but the results from other laboratories[15] seem to show that the spontaneous activation on standing does not require the presence of streptokinase. It seems thus possible, that the streptokinase preparations, though highly active against human and other sera, were devoid of any stimulating effect on the rabbit profibrinolysin, both from normal and from hypersensitized animals[12].

b. Activation by chloroform

With the aid of chloroform activation a slight activity could be obtained also in the control rabbit serum, but the activation found in the strongly hypersensitized animal (No I) was considerably higher.

The enzymic activity of the chloroform-extracted serum disappeared rather quickly[14,15] (Table 1), the test being negative when repeated 48 hours later (storage at +4°).

c. Activation by specific antigen

A sample of the euglobulin obtained from the serum of the other horse-serum-injected rabbit (No II), mixed and incubated for 30 minutes with 1/5 of its volume of horse serum before precipitation of the euglobulins, did not show any distinct activity in the viscosimetric test. This result cannot, however, be considered as conclusive and incongruent with the results of UNGAR and MIST[7], for the degree of hypersensitization was in that case probably only a slight one, when judged on the basis of the outcome of the skin test (see above).

d. Effect of protamins

Freshly prepared euglobulin (serum 1/III) showed no activity after treatment with 0.1 mg sturin sulfate per ml euglobulin solution. A slight activity obtained with salmin sulfate (same dosage used) was probably caused by a spontaneous activation on standing, another sample of the same euglobulin preparation showing a still higher activity after additional 24 hours in the refrigerator, without any addition of a protamin. The slight activity obtained with salmin sulfate in spite of its precipitating effect on the serum globulins can be interpreted as showing the non-identity of the enzyme with the major part of the serum euglobulins — the serum fraction to which it normally seems confined.

Conclusions

Summarizing the results so far obtained we may say that the hypersensitization of the type tested seems to have a certain positive effect on the potential fibrinolysin activity of the rabbit serum, as shown by the viscosimetric technique with gelatin as substrate. The highest activities — of the same order as those obtained with streptokinase-activated human euglobulin, were obtained by allowing the aging of the euglobulin fraction (i.e. by spontaneous activation after removal of the inhibitor present in the albumin fraction[14]) or by chloroform extraction of the serum of the rabbit rendered hypersensitive by intravenous injection of high doses of horse serum.

The bearings of these results on the interpretation of the role of proteolysis in the rheumatic fever type of hypersensitivity will be discussed in a subsequent paper of this series.

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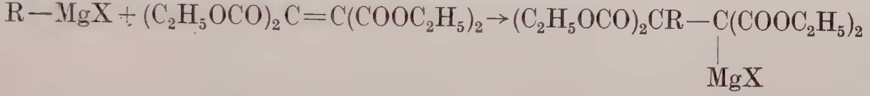
On *tert*-butylsuccinic acid and related compounds

By SIGVARD WIDEQVIST

tert-Butylsuccinic acid has not been previously described in the literature, presumably because of the difficulties in introducing *tert*-alkyl groups into chemical compounds.

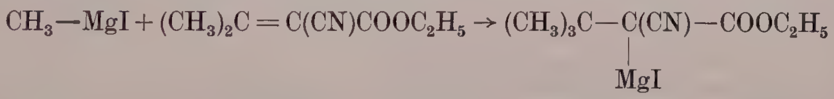
In 1949 CHI-YI HSING and LIEN-TANG LI (1) described a method for the synthesis of substituted succinic acids by the addition of Grignard reagents to ethyl ethylenetetracarboxylate



$$\mathrm{R{-}MgX + (C_2H_5OCO)_2C{=}C(COOC_2H_5)_2 \rightarrow (C_2H_5OCO)_2CR{-}\underset{\underset{MgX}{|}}{C}(COOC_2H_5)_2}$$

On decomposing the addition product with water, an *alkylated* ethyl ethanetetracarboxylate was obtained which, on hydrolysis, yielded an alkylsuccinic acid. This method seemed to be useful for the preparation of *tert*-butylsuccinic acid. However, no *tert*-butylsuccinic acid could be obtained in this way, as the reaction product was merely *unsubstituted* ethyl ethanetetracarboxylate. Evidently the Grignard reagent acted only as reducing agent.

Another way to get *tert*-butylsuccinic acid would be to condense ethyl *tert*-butylcyanoacetate and ethyl chloroacetate, whereby diethyl *tert*-butylcyanosuccinate would be obtained. Hydrolysis of the reaction product would yield *tert*-butylsuccinic acid. *tert*-Butylcyanoacetic ester is easily obtained according to a method similar to that which was indicated by the author (2) for the preparation of *tert*-butylmalonic ester, namely by adding methyl magnesium iodide to ethyl *iso*-propylidenecyanoacetate and decomposing the addition product with water:



$$\mathrm{CH_3{-}MgI + (CH_3)_2C = C(CN)COOC_2H_5 \rightarrow (CH_3)_3C{-}\underset{\underset{MgI}{|}}{C}(CN){-}COOC_2H_5}$$

This reaction gives an excellent yield. Ethyl *iso*-propylidenecyanoacetate is conveniently obtained from ethyl cyanoacetate and acetone according to WIDEQVIST (3).

The reaction between ethyl *tert*-butylcyanoacetate and ethyl chloroacetate was first performed by means of sodium ethoxide in ethyl alcohol. The yield of diethyl *tert*-butylcyanosuccinate was, however, only 27 per cent of the theoretical. About 50 per cent of the ethyl *tert*-butylcyanoacetate was recovered unchanged. Presumably the sodium salt of the *tert*-butylcyanoacetate is largely alcoholyzed to free ester and sodium ethoxide, and the sodium ethoxide rapidly consumes the chloroacetic ester. According to DEWAR (4) the dissociation constant of ethyl alcohol is 10^{-18} and that of *tert*-butyl alcohol 10^{-19}. For that reason it would be likely that the use of sodium or preferably potassium *tert*-butoxide in *tert*-butyl alcohol as condensing agent would give a better yield of the succinic ester. This proved to be the case. By using potassium *tert*-butoxide the yield of the desired condensation product increased to 55 per cent. Finally ethyl bromoacetate was substituted for the ethyl chloroacetate, whereupon the diethyl *tert*-butylcyanosuccinate was obtained in a yield of 65 per cent. The diethyl ester was converted into the monoester by selective hydrolysis with sodium hydroxide in absolute ethyl alcohol. As the monoester is a solid compound, it was purified by recrystallization from a mixture of benzene and ligroin. It was then completely hydrolyzed by boiling with concentrated hydrochloric acid, whereby *tert*-butylsuccinic acid was obtained.

Experimental

The reaction between *tert*-butyl magnesium bromide and ethyl ethylenetetracarboxylate

tert-Butyl magnesium bromide was prepared from 2.4 g of magnesium and 13.6 g of *tert*-butyl bromide in 25 ml of dry ether. Then 6.3 g of ethyl ethylenetetracarboxylate was added, and the mixture was boiled for twenty minutes. After cooling with ice, the addition product was decomposed with ice-cooled dilute sulfuric acid. The ethereal layer was separated and dried with anhydrous magnesium sulfate. On evaporating the ether, 5 g of white crystals were obtained. The product was recrystallized from ligroin and melted at 75–76°. An authentic specimen of ethyl ethanetetracarboxylate had the same melting point; when mixed with the reaction product no depression of the melting point was obtained.

Ethyl *tert*-butylcyanoacetate

$$(CH_3)_3C—CH(CN)—COOC_2H_5$$

Methyl magnesium iodide was prepared from 7.2 g (0.3 gram atom) of magnesium turnings and 42.6 g (0.3 mole) of methyl iodide in 75 ml of dry ether. A solution of 38.3 g (0.25 mole) of ethyl *iso*-propylidenecyanoacetate in 50 ml of dry ether was added to the Grignard reagent under external cooling with ice water. When all the ester was added, the reaction was completed by boiling the mixture for ten minutes. The addition product was decomposed with ice water and the precipitate of basic magnesium salt was dissolved in dilute sulfuric acid. The ethereal layer was separated and the water extracted with benzene. The combined ether and benzene solutions were shaken with a concentrated solution of sodium bisulfite in order to remove any unchanged unsaturated ester, and then with water. After drying with anhydrous sodium sulfate the solvent was distilled off and the residue fractionated *in vacuo* through a Widmer column. Ethyl *tert*-butylcyanoacetate was collected at 93–93.5°/8 mm. Yield 31.7 g (75 %).

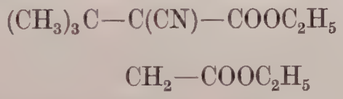
tert-Butylcyanoacetic acid

$(CH_3)_3C-CH(CN)\quad COOH$

Eight grams of ethyl *tert*-butylcyanoacetate was dissolved in 30 ml of alcohol containing 3 g of potassium hydroxide. The mixture was kept at room temperature for two days. The solvent was distilled off under reduced pressure and the residue was dissolved in water. The solution was acidified with dilute hydrochloric acid and the *tert*-butylcyanoacetic acid extracted with ether. On evaporating the ether, the acid was obtained as a white crystalline mass. It was recrystallized from benzene. M. p. 98–99°.

Anal. Subs., 0.1563 g: 9.70 ml of 0.1143-N NaOH.
» 17.05 mg: CO_2, 37.34 mg; H_2O, 12.42 mg.
» 20.51 mg: CO_2, 44.40 mg; H_2O, 14.83 mg.
» 47.82 mg: N_2, 4.075 ml at 20.0° and 759.6 mm.
Calcd. for $C_7H_{11}O_2N$ Equ. w., 141.1; C, 59.55; H, 7.85; N, 9.92.
Found Equ. w., 141.0; C, 59.77, 59.19; H, 8.15, 8.09; N, 9.93.

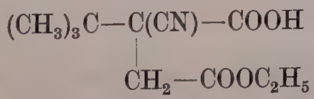
Diethyl *tert*-butylcyanosuccinate



$$\begin{array}{l}(CH_3)_3C-C(CN)-COOC_2H_5\\ \qquad\qquad\quad CH_2-COOC_2H_5\end{array}$$

In a 500-ml two-necked round-bottomed flask, fitted with a dropping funnel and a reflux condenser bearing a calcium chloride tube, were placed 80 g of absolute *tert*-butyl alcohol and 4.76 g (0.12 gram atom) of potassium. The mixture was refluxed until all the potassium was dissolved. When the solution had cooled to room temperature, 21 g (0.12 mole) of ethyl *tert*-butylcyanoacetate was added. The flask was then immersed in cold water and 20 g (0.12 mole) of ethyl bromoacetate was added drop by drop through the funnel. Potassium bromide began to separate immediately. When all the bromoester was added, the reaction mixture was kept at room temperature over night and then refluxed for two hours. The *tert*-butanol was distilled off and the residue treated with water containing some sulfuric acid, and the ester was extracted with benzene. After drying with anhydrous sodium sulfate the benzene was distilled off and the residue fractionated under reduced pressure. Diethyl *tert*-butylcyanosuccinate was collected at 167–170°/11 mm. Yield 20 g (65 %). The ester is a viscous oil.

Monoethyl *tert*-butylcyanosuccinate

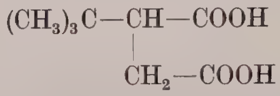


$$\begin{array}{l}(CH_3)_3C-C(CN)-COOH\\ \qquad\qquad\quad\mid\\ \qquad\qquad\quad CH_2-COOC_2H_5\end{array}$$

Sodium (1.8 g) was dissolved in 25 ml of absolute alcohol and 1.6 g of water was added. The warm solution was rapidly mixed with a solution of 20 g of diethyl *tert*-butylcyanosuccinate in 20 ml of alcohol. The sodium salt of the monoester immediately separated as a solid cake. The mixture was kept at room temperature for several hours. The sodium salt was then filtered off with suction and washed with ether. After drying, the salt was dissolved in water and the solution extracted with ether in order to remove unchanged diethyl ester. On acidifying the solution, the monoester separated as an oil which soon crystallized. Yield 13 g. It was recrystallized from a mixture of benzene and ligroin. M. p. 103.5°.

Anal. Subs., 21.59 mg: CO_2, 45.77 mg; H_2O, 14.64 mg.
Calcd. for $C_{11}H_{17}O_4N$ C, 58.11; H, 7.54.
Found C, 57.81; H, 7.59.

tert-Butylsuccinic acid



$$\begin{array}{lll}(CH_3)_3C- & CH & -COOH\\ & | & \\ & CH_2 & -COOH\end{array}$$

Five grams of monoethyl *tert*-butylcyanosuccinate was boiled in a reflux apparatus with 50 ml of concentrated hydrochloric acid for twenty-three hours. After cooling, the solution was extracted three times with ether. The extract was dried over anhydrous magnesium sulfate. On evaporating the ether, an oil was obtained which, on scratching, solidified to white crystals. Yield 3 g of *tert*-butylsuccinic acid. It is readily soluble in water. It was recrystallized by dissolving in warm water. The solution was cooled in ice and saturated with hydrogen chloride, causing the acid to separate as white crystals. The crystals were filtered off, washed with a little concentrated hydrochloric acid and dried *in vacuo* over caustic potash. M. p. 132°.

Anal. Subs., 23.01 mg: CO_2, 46.77 mg; H_2O, 16.48 mg.
Calcd. for $C_8H_{14}O_4$ C, 55.14; H, 8.11.
Found C, 55.42; H, 8.01.

SUMMARY

Ethyl *tert*-butylcyanoacetate was prepared in a yield of 75 % by adding methyl magnesium iodide to ethyl *iso*-propylidenecyanoacetate. B. p. 93–93.5°/8 mm. Diethyl *tert*-butylcyanosuccinate was obtained in a yield of 65 % by condensing ethyl *tert*-butylcyanoacetate and ethyl bromoacetate by means of potassium *tert*-butoxide in *tert*-butanol. B. p. 167–170°/11 mm. The monoethyl *tert*-butylcyanosuccinate was prepared by selective hydrolysis of the diethyl ester. M. p. 103.5°. On boiling the monoester with conc. hydrochloric acid for twenty-three hours, *tert*-butylsuccinic acid was obtained. M. p. 132°.

Laboratory of Organic Chemistry, University of Uppsala.

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A crystal structure investigation of the paramolybdate ion

By Ingvar Lindqvist

With 12 figures in the text

In the course of our investigations on the crystal chemistry of the polymolybdates, the structure of the paramolybdate ion in ammonium paramolybdate, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ has been determined. A short communication of the structure has been published recently (1), and a full report of the structure determination is now given.

Crystals

The crystals were prepared by recrystallization from a water solution of a commercial ammoniumparamolybdate (Grave, Stockholm). Most of the crystals were rhomboidal plates (cf. Sturdivant (2)), but we also obtained some crystals of a more needle-shaped form. In the notation of Sturdivant (2) the needle axis was parallel to the a-axis. To avoid complications due to absorption we preferred working with these needles. The formula of the salt is $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$, (2, 3). The unit cell dimensions are $a = 8.382$ Å, $b = 36.125$ Å, $c = 10.464$ Å and $\beta = 116°0'$. There are four units $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ in the cell. The space-group is C_{2h}^5—$P\,2_1/c$. (All data from (2)).

Single crystal photographs

It would have been desirable to work with Mo-$K\alpha$ radiation, but the large b-axis, 36 Å, would then make an accurate indexing very difficult. Even with the Cu-K radiation used, the indexing was not easy to perform. Weissenberg photographs were taken around the a-axis (the layer lines 0–4). One Weissenberg oscillation photograph (cf. (5)) was used to correlate the intensities of the different Weissenberg photographs. The intensities were estimated visually, starting from the relative values for the intensities of Cu-$K\alpha_1$, Cu-$K\alpha_2$ and Cu-K_β radiation. To be sure of perfect indexing and the best possible estimation of the intensities, the procedure was carried out twice. In this way we got the intensities of 621 reflections. The intensity values were multiplied by $\cos^2 \mu \cdot \sin \Upsilon/(1+\cos^2\Theta)$ using the curves of Chia-Si-Lu (6). Fourier summations were carried out by means of the Hägg-Laurent machine (7) and, for frequencies higher than 16 with the Robertson strips (8).

Molybdenum positions

The most natural way to attack the problem is no doubt to try to determine the molybdenum positions. This seems very easy at the first sight. The space-group

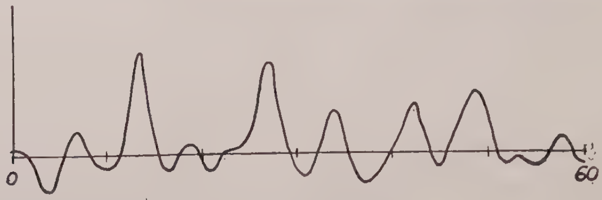

Fig. 1. P(0, y, ½).

$P\ 2_1/c$ has the following general position 4(e): x, y, z; $\bar{x}$, y, z; $\bar{x}$, ½+y, ½−z; x, ½−y, ½+z. It is not possible to work with Patterson projections, because the large axes make projections as p(xz), p(y) too complicated. This will become still more evident after the following report of the work with P(x, y, z) cuts. The coordinates of the general position indicate, however, two very important cuts. First we have P(0, y, ½), which will give values of ½—2 y_n, if (x_n, y_n, z_n) are the parameters of the general position occupied by the n:th molybdenum atom. From P(x, ½, z), on the other hand, maxima of the type (2 x_n, 2 z_n—½) will be obtained. P(0, y, ½) is given in Fig. 1 and P(x, ½, z) in Fig. 2). (Only a quarter of the Patterson space is considered: x = 0–60/60, y = 0–60/120, z = 0–30/60). In the order of their heights the maxima in P(0, y, ½) are situated at y = 13.5/120, y = 27/120, y = 48.5/120, y = 42/120, y = 34/120, y = 6.5/120, y = 57.5/120 and y = 18.5/120. The two last maxima are, however, so small that they could not possibly correspond to Mo-Mo distances. The other six give the following y parameters, possible: y_1 = 23.25/120, y_2 = 16.5/120, y_3 = 5.75/120, y_4 = 9/120, y_5 = 13/120 and y_6 = 26.75/120. The maxima giving y_1 and y_2 are larger than the others, and may each correspond to two Mo-Mo distances. In that case the smallest maximum, giving y_6, should not be considered. The answer to these problems can only be given later. We may notice that y_1 = 23.25/120 also means that we have y-values 36.75/120, 83.25/120 and 96.75/120.

Then the cut P(x, ½, z) (Fig. 2) will be discussed, where the maxima are denoted by the letters A–G. Most of these maxima are irregular and diffuse. The E maximum, which is rather sharp, is situated at x = 21/60 and z = 18.5/60. These values correspond to $2x_E$ = 21/60 and $2z_E$—½ = 18. 5/60,and thus to x_E = 10.5/60 and z_E = 24.25/60. We must notice, however, that it also corresponds to all the four (x, z) which form a general position, that is ($\overline{10.5}$/60, $\overline{24.25}$/60), ($\overline{10.5}$/60, 5.75/60) and (10.5/60, $\overline{5.75}$/60). We also find that the coordinates $x'_n = x_n + \frac{1}{2}$, $z'_n = z_n$, (40.5/60, 24.25/60), (19.5/60, $\overline{24.25}$/60), (19.5/60, 5.75/60) and (40.5/60, $\overline{5.75}$/60) are in agreement with the maximum E. (None of these values must be taken as accurate and far less any derived from the other maxima.) If we decide to treat only y values between 0 and 30/120 (which is possible, because positions with other y values give no new arrangements), therefore for each y value we must choose one of the eight pairs of (x, z) parameters of one of the seven possible (x, z) octets. Of course, the first choice of a pair of the first octet is arbitrary, but then the following parameters must be chosen in agreement with the first (x, z) pair. This is the reason why the structure determination becomes so complicated when there are as many as seven independent general positions of the heavy atoms, in this case molybdenum. If we assume that each maximum in P(x, ½, z) corresponds to a Mo atom and as above that the five first y values from P(0, y, ½) correspond to seven different Mo atoms with two atoms in y_1 and y_2, these (x, z) and y values could be permuted in more than 10^{11} inde-

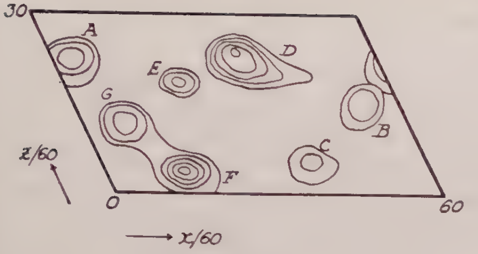

Fig. 2. P(x, $\frac{1}{2}$, z).

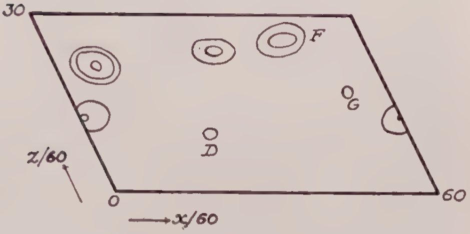

Fig. 3. P(x, 46/120, z).

pendent ways, and even if we had made a proper choice of y for each (x, z) octet, the (x, z) values could be chosen in more than 10^8 different ways.

This great number of possibilities may be greatly reduced, however, by considering a three-dimensional Patterson synthesis, P(x, y, z). We have therefore calculated P(x, 2n/120, z) for each n from 0 to 30. Before the discussion of the results of this study, we must notice that there are two kinds of maxima corresponding to Mo-Mo distances in P(x, y, z). We have already studied one kind, those vectors which join Mo atoms belonging to the same general position, here called *equivalent* vectors. But there are also vectors joining Mo atoms with different general positions, here called *independent* vectors. It is worth mentioning that, theoretically in $P\,2_1/c$ every independent vector is repeated twice as many times in the whole crystal as an equivalent vector. Therefore, the latter should give only half as high maxima, if they were based on an absolute scale. In any case they should be lower than the independent vector maxima. If this fact is overlooked, it is very difficult to explain the P(x, y, z) cuts.

At first we use P(x, y, z) to find possible (x, z) octets for each y value. This is performed by searching in the cuts P(x, $2y_n$, z) for vectors [$2x_n$, $2y_n$, $2z_n$], which depend on the centro-symmetry. We begin with $y_1 = 23.25/120$ investigating P(x, 46.5/120, z). This cut is not contained in P(x, 2n/120, z) but P(x, 46/120, z) (Fig. 3) will give all the information desired. The maxima, which agree with any ($2x_n$, $2z_n$) calculated from P(x, $\frac{1}{2}$, z) are denoted quite in the same way as the original maxima in P(x, $\frac{1}{2}$, z). We notice the following (x, z) octets, which possibly could be attributed to $y = 23.25/120$: F, D, and G. F is the largest maximum, D does not correspond to the centre of the D maximum in P(x, $\frac{1}{2}$, z), but to a point in the elongated part of the maximum. There cannot be more than two Mo atoms with $y = 23.25/120$, and it is not possible yet to decide which one or which two of the octets F, D, and G should be chosen. The possible octets for the other y values are here given without the P(x, 2n/120, z) diagrams. (Doubtful agreement is marked with ?.)

$y_2 = 16.5/120$	P(x, 33/120, z) gives	D, A, F, G (?)
$y_3 = 5.75/120$	P(x, 11.5/120, z) gives	E, A (?)
$y_4 = 9/120$	P(x, 18/120, z) gives	F, G and C
$y_5 = 13/120$	P(x, 26/120, z) gives	F, B
$y_6 = 26.75/120$	P(x, 53/120, z) gives	F (?), G (?).

The poor agreement for F and G in the last cut together with the smallness of the y_6 maximum in P(0, y, $\frac{1}{2}$) makes it unnecessary to discuss further the possibility of a Mo atom placed in y_6. The problem then remains to choose two of the possible octets for each of y_1 and y_2 and one for each of y_3, y_4, and y_5. Besides, the correct (x, z) pair should be fixed in each octet.

These questions probably could be completely answered by a thorough discussion of all P(x, 2n/120, z) cuts, placing the Mo atoms one after the other. We started in this way, but later used a more rapid procedure to get the correct parameters of all seven Mo atoms. At first, we shall here resume the discussion of the primary attack on the problem. As starting point we chose an E pair in y_3, where the agreement of E is far better than that of A. The coordinates (x, y, z) thus arbitrarily fixed were (19.5/60, 5.75/120, 5.75/60) (point II). As there are only B and F to decide between in y_5, we tried to combine a pair from any of these two octets in y_5 with the original point II to get a new point I. For this purpose we must especially study the cuts determined by all y components (in the range 0–60/120) arising from combination of y_3, $\frac{1}{2}-y_3$, $\frac{1}{2}+y_3$, $\bar{y}_3$ with y_5, $\frac{1}{2}-y_5$, $\frac{1}{2}+y_5$, $\bar{y}_5$. These y values are $y_3-y_5 = 7.25/120$, $\frac{1}{2}-y_5-y_3 = 41.25/120$, $\frac{1}{2}+y_3-y_5 = 52.75/120$ and $y_3+y_5 = 18.75/120$. If we index the coordinates of II (x_3, y_3, z_3) and those of I (x_5, y_5, z_5) the coordinates of the general position (x_3, y_3, z_3), $(x_3, \frac{1}{2}-y_3, \frac{1}{2}+z_3)$, $(\bar{x}_3, \frac{1}{2}+y_3, \frac{1}{2}-z_3)$, $(\bar{x}_3, \bar{y}_3, \bar{z}_3)$ and (x_5, y_5, z_5), $(x_5, \frac{1}{2}-y_5, \frac{1}{2}+z_5)$, $(\bar{x}_5, \frac{1}{2}+y_5, \frac{1}{2}-z_5)$, $(\bar{x}_5, \bar{y}_5, \bar{z}_5)$ require the following (x, z) vectors in the different cuts:

$$\begin{array}{lll}
y = y_5-y_3 & x = x_5-x_3 & z = z_5-z_3 \\
y = \frac{1}{2}-y_5-y_3 & x = x_5-x_3 & z = \frac{1}{2}+z_5-z_3 \\
y = \frac{1}{2}+y_3-y_5 & x = \bar{x}_5+\bar{x}_3 & z = \frac{1}{2}-z_3-z_5 \\
y = y_3+y_5 & x = x_3+x_5 & z = z_3+z_5.
\end{array}$$

In all these discussions we must keep in mind that only one quarter of the Patterson space is studied, namely x = 0–60/60, y = 0–60/120 and z = 0–30/60. This is permissible, because the cuts $P(x, y_n, z)$ and $P(x, \bar{y}_n, z)$ are identical. Maxima in the three quarters of the Patterson space not studied are already given in the calculated quarter. Thus (0.6, 0.6, 0.6), (0.6, 0.4, 0.6) and (0.4, 0.6, 0.4) are all identical with (0.4, 0.4, 0.4).

Now all information for $y = y_5-y_3 = 7.25/120$ could be derived from the nearest cut calculated, namely P(x, 8/120, z) (fig. 4). The maxima marked E–B may correspond to B (3/60, 13/120, 7.5/60) or B (33/60, 13/120, 7.5/60), the one marked B–E to B (27/60, 13/120, 22.5/60). The latter may also correspond to F (7.5/60, 13/120, 13.5/60). The next cut to study is P(x, 42/120, z) (Fig. 5) giving $y = \frac{1}{2}-y_5-y_3 = 41.25/120$. Here we only search for vectors corresponding to those already found in Fig. 4. The sole vector which fulfills these conditions, B–E, is in agreement with B(27/60, 13/120, 22.5/60). To be sure we ought to investigate also $y = \frac{1}{2}+y_3-y_5$ and $y = y_3+y_5$. As a matter of fact these cuts P(x, 52/120, z) and P(x, 18/120, z) (Fig. 6 and 7) show maxima, marked B–E, corresponding to B (27/60, 13/120, 22.5/60) (I). As all interatomic vectors II–I calculated are evidently found in P(x, y, z), we state that two Mo atoms have the positions II and I in the unit cell. (This conclusion is not quite unequivocally based, but can be considered as correct until contradictions arise.) These two y values are the easiest ones to treat, but probably it should be possible to go on in this way, even with the more intricate ones. In an analogous way we found that in y_2 for A(31.5/60, 16.5/120, 4.5/60), (VI), all calculated interatomic distances VI–II and VI–I are found in P(x, y, z). This mode of reasoning was discontinued, however, and another method tried.

We have earlier stated, that the Patterson projections were of no value for finding

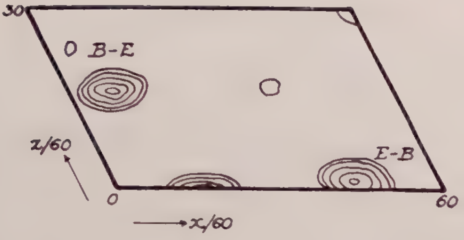

Fig. 4. P(x, 8/120, z).

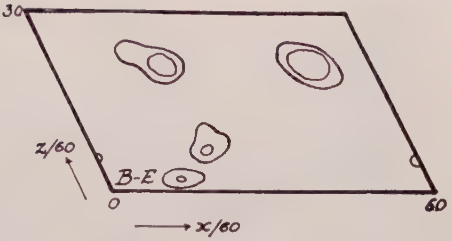

Fig. 6. P(x, 52/120, z).

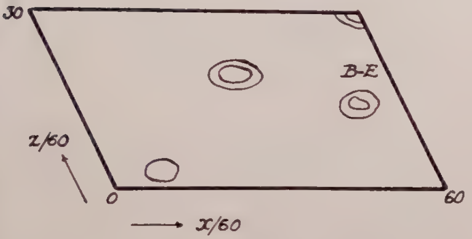

Fig. 5. P(x, 40/120, z).

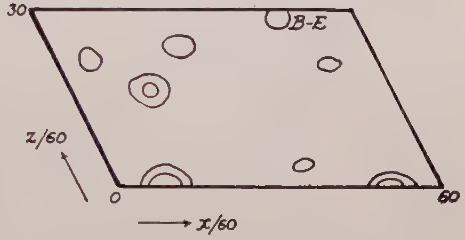

Fig. 7. P(x, 18/120, z).

the structure, and the rather intricate determination of the three first Mo parameters from Patterson cuts gives evidence to this statement. Electron density projections on the other hand are not nearly as complicated, so we decided to try to calculate an electron density projection $\rho(y, z)$, based on the three points determined. We hoped to find these points confirmed, and eventually to find new (y, z) pairs. In the determination of the signs of the structure factor of 0 *k l*, the sign calculated was taken as correct if $\Sigma \cos 2\pi (ky_n + lz_n)$ was larger than 0.7. No other F values were used in the calculation of $\rho(y, z)$. The result (Fig. 8) is rather surprising. The three points, I, II and VI are very well confirmed, and three other high new maxima have appeared. The highest of these is marked III, and is in agreement with D in y_1. We then made the assumption that we really had another Mo atom in III, recalculated the signs of F (0 *k l*) and got a new $\rho(y, z)$. In this projection we also chose the highest extra maximum as a new (y, z) position and repeated the procedure until we did not get any new growing maxima. Then there are seven maxima in $\rho(y, z)$ (Fig. 9), quite in agreement with the number of Mo atoms in the unit cell. Before we confirm these parameters in another way, it seems reasonable to study the above procedure a little more.

Two questions especially arise, namely, whether it is possible to get a wrong position confirmed in this way, and whether we could get a new correct position even if there are some errors in the original positions. Therefore, we recalculated the signs starting with I, II, and a wrong position $y = 30/120$, $z = 15/60$. The resulting $\rho(y, z)$ is given in Fig. 10. We notice that of the original points, I and II are well established, but the wrong position is not obtained. Further the maxima VI, III and V have appeared although we have used one false point in the calculation of the signs. Of course this little study is not at all conclusive, but we think that it is worth indicating this possibility of making a rather fast parameter determination in cases with many heavy atoms in a large cell. If only high maxima are considered there

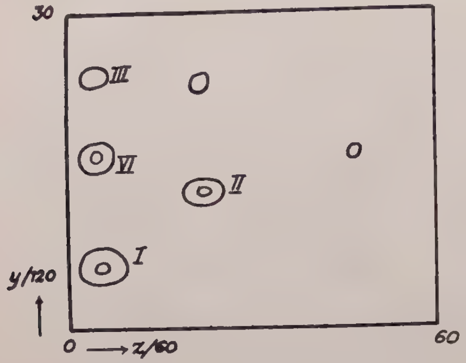

Fig. 8. ρ(y, z) preliminary.

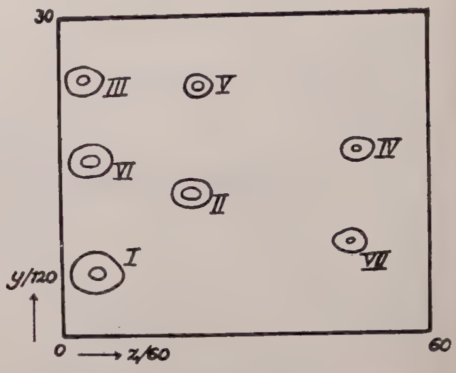

Fig. 9. ρ(y, z) final.

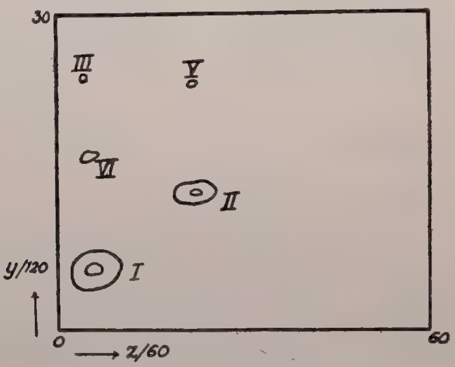

Fig. 10. ρ(y, z) error study.

seems to be little risk of getting false new parameters. In any case these can be checked by a three-dimensional synthesis, P(x, y, z).

It then remained to determine the x parameters of the seven Mo atoms. We proceeded in quite the same way with (x, y) as with (y, z) above. Going out from I, II and VI, calculating the signs of F(h k 0), we obtained the electron density projection ρ(x, y). The maxima of ρ(x, y) were more diffuse than those of ρ(y, z) (h is only 0–4), but even here seven final positions were determined. The complete parameters thus obtained were

I	x = 27.5/60	y = 13.5/120	z = 22.5/60
II	x = 19.0/60	y = 6.0/120	z = 5.5/60
III	x = 11.0/60	y = 23.5/120	z = 5.0/60
IV	x = 4.0/60	y = 16.5/120	z = −10.5/60
V	x = 34.0/60	y = 23.5/120	z = 21.5/60
VI	x = 30.5/60	y = 16.5/120	z = 4.5/60
VII	x = 19.0/60	y = 9.0/120	z = −12.0/60.

In the course of the structure determination, we have already shown which equivalent and independent vectors could be derived from a given set of general positions

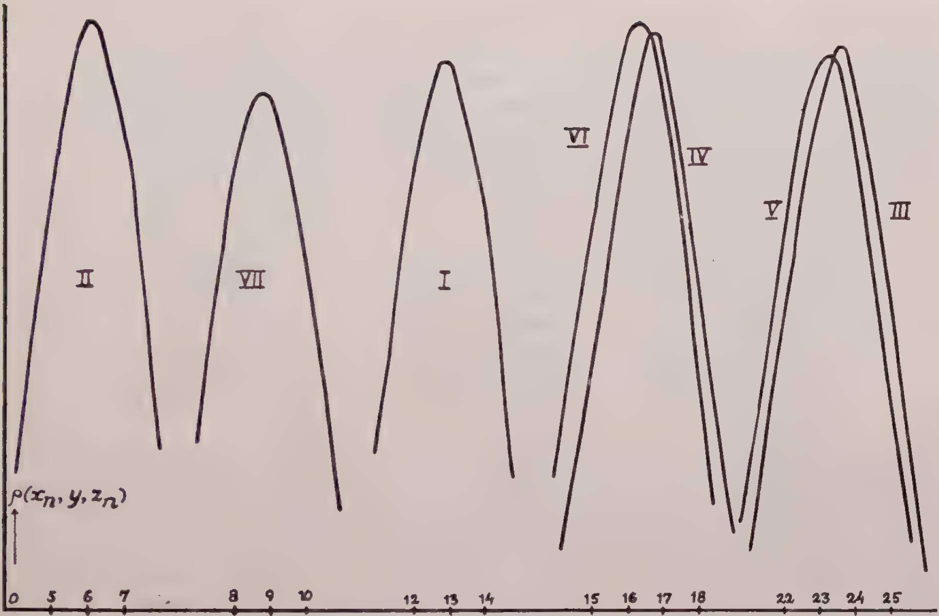

Fig. 11. $\rho(x_n, y, z_n)$ for the seven different (x_n, z_n).

(cf. the derivation of II and I). We have also remarked that the structure determination carried out is not unequivocally conclusive to the same degree as has sometimes been possible (cf. (9)), especially because there are so many ways to combine the (x_n, z_n) pairs with y_n. As we had a complete $P(x, 2n/120, z)$ it seemed suitable to investigate whether all independent and equivalent vectors calculated were confirmed in $P(x, 2n/120, z)$ (cf. the much easier confirmation in (9), using only $P(x, 0, z)$, $P(x, \frac{1}{4}, z)$ and $P(x, \frac{1}{2}, z)$). The above seven positions should give rise to 84 independent and 21 equivalent vectors in a quarter of the Patterson space. They were all identified in their correct places. (Discrepancies amounting to 3/60 were obtained in x, which naturally has the least accurate values, due to the small number $h_{\max} = 4$.) Besides, there are only two really high maxima left which could not be explained as such vectors and about ten of the same altitude as the smallest vectors calculated. This seems to be a very convincing confirmation of the (x, y, z) parameters obtained. As we found it valuable, however, for other reasons, to get more accurate values of x, y, and z, we carried out a three-dimensional electron density calculation, $\rho(x, y, z)$, which gave further evidence.

In computing the signs of all $F(h\,k\,l)$, we proceeded as with $F(0\,k\,l)$, taking care only of the trigonometric factors of $F(h\,k\,l) = \Sigma f_n \cos 2\pi(hx_n + ky_n + lz_n)$. Therefore, the calculated values could not be directly compared with the estimated F-values but the agreement was good between high values of $\Sigma \cos 2\pi(hx_n + ky_n + lz_n)$ and of $F(h\,k\,l)$ observed. Of the 621 F values not more than 51 were left as doubtful as to their signs even at the first trial. We then calculated $\rho(x, y_n, z)$ for all y_n found, and obtained seven (x_n, z_n) maxima in clear agreement with the original ones, but with some small displacements in (x, z). A new sign calculation left only 14 signs in doubt. As the *b*-axis is so large, we tried to determine the y values more accurately by calculating $\rho(x_n, y, z_n)$ for each (x_n, z_n) pair. The results are given in Fig. 11 showing the degree of agreement between the altitudes of the seven y peaks. Of course it should have been possible to repeat these procedures, but our observations, while the calculations were being carried out, indicated that one could not hope to get more accurate parameters from the experimental data in this way. The errors are treated in the later discussion of the structure of the paramolybdate ion. The final parameters were:

I	$x = 0.450$	$y = 0.107$	$z = 0.367$
II	$x = 0.317$	$y = 0.051$	$z = 0.097$
III	$x = 0.175$	$y = 0.196$	$z = 0.087$
IV	$x = 0.058$	$y = 0.139$	$z = -0.183$
V	$x = 0.567$	$y = 0.192$	$z = 0.353$
VI	$x = 0.475$	$y = 0.137$	$z = 0.075$
VII	$x = 0.317$	$y = 0.074$	$z = -0.203$

The structure factors were not calculated for all F(*h k l*) from these parameters, because the 6 NH_4^+, 24 O^{2-} and 4 H_2O may also give at least small contributions to F(*h k l*) and therefore a comparison between observed and calculated F-values is not nearly as conclusive as our previous investigation of P(x, y, z). To show the crude but clear agreement, however, we have calculated F(0 *k l*). The results are given in Table 1. We must notice that the effect of the inhomogeneous absorption, caused by the irregular shape even of the approximately needle-shaped crystals, is not considered in this calculation. The absorption factor will always influence the F values of a lot of neighbouring indices but never the F value of an isolated *h k l*. Nor is the temperature factor considered, which will make our calculated F values too large for high numbers of *h*, *k* and *l*.

In conclusion the positions of the seven molybdenum atoms must be stated as definitely determined.

Note on the threedimensional Patterson cuts

There are some facts in connection with P(x, y, z) which ought to be discussed further. As already stated there were two really high maxima left after considering all interatomic Mo-Mo vectors. One of these maxima is situated at (30/60, 0,0). This maximum is easily explained by the fact that the threedimensional summation is only carried out for $h = 0$–4. All $F^2(h\,k\,l)$ with even *h* contribute with positive values and those with odd *h* with negative values in this point. As we thus use the three even *h* values, 0, 2 and 4 and only two odd *h* values, 1 and 3, it is quite natural that we get a rather sharp extra maximum in (30/60, 0, 0). One may also notice that the maxima in P(x, 60/120, z) (Fig. 2) and also in P(x, 0, z) are higher than for example those in P(x, 46/120, z) (Fig. 3). For every (x, y_n, z) maximum, however, we have an identical maximum in (x, y_n, z). These coincide for $y_n = 0$ and $y_n = 1/2$, and therefore the maxima in P(x, 0, z) and P(x, 60/120, z) attain higher values than the maxima in other P(x, y, z) cuts.

Structure of the paramolybdate ion. Oxygen positions

The next problem was to find a proper oxygen configuration around the seven Mo atoms. We then had to use the information about the Mo–O coordination and the

Table 1.

Comparison between observed and calculated relative |F (0 *k l*)| values.

0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.
0 2 0	—	0	0 36 1	—	1	0 16 3	2	3	0 32 4	—	2
0 4 0	8	6	0 0 2	—	2	0 17 3	2	1	0 33 4	—	0
0 6 0	—	0	0 1 2	—	1	0 18 3	—	1	0 34 4	—	2
0 8 0	4	4	0 2 2	—	2	0 19 3	—	3	0 35 4	5	6
0 10 0	—	1	0 3 2	—	2	0 20 3	—	3	0 36 4	4	4
0 12 0	9	7	0 4 2	—	2	0 21 3	—	1	0 1 5	—	1
0 14 0	—	3	0 5 2	3	3	0 22 3	4	4	0 2 5	—	2
0 16 0	5	7	0 6 2	3	2	0 23 3	—	4	0 3 5	—	0
0 18 0	6	6	0 7 2	4	5	0 24 3	—	0	0 4 5	—	1
0 20 0	6	5	0 8 2	—	1	0 25 3	—	1	0 5 5	2	4
0 22 0	—	2	0 9 2	3	3	0 26 3	—	0	0 6 5	1	4
0 24 0	3	5	0 10 2	—	0	0 27 3	—	1	0 7 5	—	3
0 26 0	—	2	0 11 2	2	2	0 28 3	—	1	0 8 5	4	4
0 28 0	—	1	0 12 2	4	3	0 29 3	—	1	0 9 5	—	2
0 30 0	—	2	0 13 2	4	4	0 30 3	—	1	0 10 5	—	2
0 32 0	6	5	0 14 2	2	2	0 31 3	—	2	0 11 5	—	2
0 34 0	7	7	0 15 2	—	2	0 32 3	—	0	0 12 5	6	6
0 36 0	7	7	0 16 2	—	0	0 33 3	—	0	0 13 5	6	4
0 1 1	7	8	0 17 2	—	1	0 34 3	5	5	0 14 5	—	0
0 2 1	3	3	0 18 2	—	1	0 35 3	—	0	0 15 5	—	1
0 3 1	—	2	0 19 2	—	2	0 36 3	—	3	0 16 5	6	3
0 4 1	4	4	0 20 2	—	2	0 0 4	5	9	0 17 5	—	3
0 5 1	—	1	0 21 2	—	1	0 1 4	5	9	0 18 5	—	1
0 6 1	4	5	0 22 2	—	1	0 2 4	—	1	0 19 5	—	2
0 7 1	—	1	0 23 2	—	2	0 3 4	3	5	0 20 5	2	2
0 8 1	2	1	0 24 2	—	0	0 4 4	—	2	0 21 5	4	4
0 9 1	2	4	0 25 2	7	5	0 5 4	—	2	0 22 5	—	2
0 10 1	1	1	0 26 2	—	1	0 6 4	—	1	0 23 5	—	1
0 11 1	6	6	0 27 2	—	2	0 7 4	—	2	0 24 5	1	4
0 12 1	7	7	0 28 2	2	3	0 8 4	—	2	0 25 5	—	0
0 13 1	—	1	0 29 2	3	2	0 9 4	—	0	0 26 5	—	0
0 14 1	4	5	0 30 2	—	0	0 10 4	—	2	0 27 5	—	4
0 15 1	4	6	0 31 2	—	2	0 11 4	—	0	0 28 5	—	3
0 16 1	3	5	0 32 2	—	2	0 12 4	5	5	0 29 5	—	3
0 17 1	—	2	0 33 2	—	1	0 13 4	7	6	0 30 5	—	2
0 18 1	3	3	0 34 2	—	1	0 14 4	4	4	0 31 5	—	1
0 19 1	—	3	0 35 2	—	0	0 15 4	—	0	0 32 5	—	2
0 20 1	—	0	0 36 2	—	1	0 16 4	3	3	0 33 5	—	0
0 21 1	4	4	0 1 3	—	2	0 17 4	7	7	0 34 5	—	0
0 22 1	6	6	0 2 3	12	11	0 18 4	—	0	0 35 5	—	1
0 23 1	3	3	0 3 3	—	2	0 19 4	6	4	0 36 5	—	1
0 24 1	—	0	0 4 3	—	0	0 20 4	6	5	0 0 6	—	1
0 25 1	—	1	0 5 3	2	2	0 21 4	—	1	0 1 6	—	0
0 26 1	—	2	0 6 3	4	2	0 22 4	—	1	0 2 6	—	0
0 27 1	2	2	0 7 3	—	1	0 23 4	—	3	0 3 6	—	2
0 28 1	—	3	0 8 3	—	0	0 24 4	—	3	0 4 6	—	1
0 29 1	—	0	0 9 3	3	4	0 25 4	—	2	0 5 6	—	2
0 30 1	4	4	0 10 3	—	0	0 26 4	—	1	0 6 6	—	0
0 31 1	—	0	0 11 3	4	5	0 27 4	—	0	0 7 6	—	2
0 32 1	—	2	0 12 3	—	0	0 28 4	—	2	0 8 6	—	1
0 33 1	—	3	0 13 3	6	6	0 29 4	—	0	0 9 6	—	1
0 34 1	—	2	0 14 3	9	7	0 30 4	—	0	0 10 6	—	1
0 35 1	—	1	0 15 3	4	3	0 31 4	4	4	0 11 6	5	5

Table 1 (cont.)

0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.	0 *k l*	Obs.	Calc.
0 12 6	—	2	0 30 6	—	0	0 12 7	—	2	0 30 7	—	2
0 13 6	2	3	0 31 6	—	0	0 13 7	—	2	0 31 7	—	2
0 14 6	—	0	0 32 6	—	0	0 14 7	—	3	0 32 7	—	0
0 15 6	—	2	0 33 6	—	1	0 15 7	5	4	0 33 7	3	5
0 16 6	—	1	0 34 6	—	0	0 16 7	—	1	0 34 7	—	4
0 17 6	—	2	0 35 6	—	0	0 17 7	—	1	0 35 7	—	0
0 18 6	—	0	0 36 6	—	1	0 18 7	—	0	0 36 7	—	2
0 19 6	—	2	0 1 7	7	6	0 19 7	—	2	0 0 8	—	1
0 20 6	—	1	0 2 7	6	5	0 20 7	—	1	0 1 8	6	4
0 21 6	—	2	0 3 7	5	5	0 21 7	—	2	0 2 8	—	1
0 22 6	—	2	0 4 7	—	1	0 22 7	—	2	0 3 8	—	0
0 23 6	—	1	0 5 7	—	0	0 23 7	—	2	0 4 8	—	2
0 24 6	—	1	0 6 7	—	1	0 24 7	—	2	0 5 8	—	2
0 25 6	—	2	0 7 7	—	1	0 25 7	—	0	0 6 8	—	2
0 26 6	—	1	0 8 7	—	1	0 26 7	—	0	0 7 8	—	2
0 27 6	—	0	0 9 7	—	1	0 27 7	—	2	0 8 8	—	2
0 28 6	—	2	0 10 7	—	1	0 28 7	—	0	0 9 8	—	0
0 29 6	—	1	0 11 7	—	1	0 29 7	—	2	0 10 8	—	2

data on interatomic distances already yielded by crystal chemistry. A survey of these coordinations and distances is given by MAGNÉLI (10). Evidently Mo has a tendency to form condensed anion polyhedra, built up by MoO_6 octahedra and sometimes MoO_4 tetrahedra. To find the coordinations in this case it was necessary to calculate all mutual interatomic distances between I–VII. It then was evident that only one of the seven Mo atoms, VI, was at approximately the same distance from the other six Mo atoms. These values, 3.29–3.48 Å, correspond without doubt to the distance between the centers of two MoO_6-octahedra sharing edges. All the other Mo atoms show such distances to only three of the other Mo atoms (values between 3.21 and 3.48 Å). A further study of the neighbourhood of VI shows that the interatomic vectors from VI are not symmetrically directed. A crude calculation of the angles between the vectors gave the following values (together with the idealized values in parentheses).

I–VI–II 58°5 (60°)
I–VI–III 80°5 (90°)
I–VI–IV 105°7 (120°)
I–VI–V 57°9 (60°)
I–VI–VII 114°7 (120°)
II–VI–IV 77°3 (90°)
II–VI–V 116°7 (120°).
II–VI–VII 55°7 (60°)
III–VI–IV 57°6 (60°)
III–VI–V 59°9 (60°)
III–VI–VII 109°8 (120°)
IV–VI–V 116°1 (120°)
V–VI–VII 170°2 (180°)

The model given in Fig. 12 a and 12 b which corresponds to the formula $Mo_7O_{24}^{6-}$ fulfills all these idealized conditions and is in all principal respects in agreement with the Mo positions. The structure could best be described as built up by four MoO_6 octahedra I–IV. I–II and III–IV share edges, I–III and II–IV share corners; thus the four centers of these octahedra form a rectangle. On this rectangle three other octahedra V–VII are placed, so that these three octahedra share edges with all

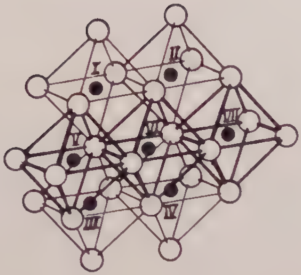

Fig. 12 a. The paramolybdate ion. Black circles molybdenum, open circles oxygen. Ideal structure with regular octahedra.

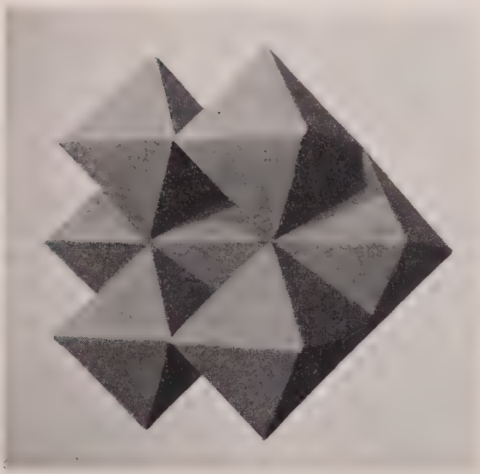

Fig. 12 b. The paramolybdate anion polyhedron. Ideal structure with regular octahedra.

their nearest neighbours (V with I, III and VI and so on). The idealized structure is distorted in the paramolybdate crystal and we will here discuss these distortions as they are manifested in the values of the length and direction of the interatomic vectors. At first we study I–III and II–IV, which correspond to shared corners, and which should be parallel. The components of the vectors are for I–III [16.5/60, 10.6/120, 16.5/60] and for II–IV [16.0/60, 10.6/120, 16.5/60] so they are practically parallel to each other. The values of the lengths are 4.30 Å and 4.27 Å, which are larger than any values reported in (10), but which are not very much different from the double Mo–O distances given there for octahedral coordination. We must not consider this sharing of corners as a primary factor determining the structure, but rather as a result of the way in which the octahedra share edges. The length 4.3 Å is then indeed too small from a geometrical point of view. (All idealized 120° angles correspond to smaller angles in the real structure.)

The Mo-Mo distances corresponding to shared edges are:

I–II	3.27 Å	II–VI	3.39 Å	III–V	3.35 Å	IV–VII	3.27 Å
I–V	3.21 Å	II–VII	3.21 Å	III–VI	3.36 Å	V–VI	3.35 Å
I–VI	3.29 Å	III–IV	3.28 Å	IV–VI	3.45 Å	V–VII	3.48 Å

It is not easy to state whether there is any reality in the deviation from the mean value 3.33 Å, which is in agreement with earlier data (3.37 Å (11) and 3.42 Å (12) from MoO_3, 3.31 Å (13) for $TeMo_6O_{24}^{6-}$). The values probably indicate that VI has been placed a little too near to I, III, and V. A displacement of VI 0.06 Å in the direction of VII makes VI–V and VI–VII almost equal, and a very small further displacement toward IV would evidently give VI a place at approximately the same distance from all the four nieghbours I–IV. It therefore seems reasonable to explain the deviations as caused by errors in the parameter determination. We then also get a measure of the error, which seems to give an inaccuracy of about 0.05 Å in the position of the Mo atoms, and therefore up to 0.1 Å in the Mo-Mo distances. Even considering this error we obtain, however, the Mo-Mo vectors originating from VI a little larger than those between other pairs of two Mo atoms, the mean values being 3.39 Å and 3.27 Å. (The value 3.31 Å from (13) corresponds to Mo-Mo distances of the last kind.) In studying the parallelism we found the following vectors to be essentially parallel: I–II and III–IV, IV–VII and III-VI, II–VI and I–V, II–VII and I–VI, III–V and IV–VI. The vectors V–VI and VI–VII show a clear deviation, the angle between them being only 170° instead of 180°. Other deviations in the angles have already been given above. All deviations are no doubt partly due to inaccuracy in the parameter determination, but they are probably also a manifestation of a distortion, which is connected especially with the difference between the two kinds of Mo-Mo distances just mentioned, of MoO_6 octahedra sharing edges. All other deviations must not be regarded as real before a knowledge of the complete crystal structure can explain them.

All oxygen positions are approximately fixed by the above determination of the structure of the paramolybdate ion $Mo_7O_{24}^{6-}$. No other experimental data can be used for the derivation of the definite oxygen positions because the influence of the oxygen atoms on the intensity of the X-ray reflexions is too small compared with that of the Mo atoms to give any conclusive quantitative effects. As the paramolybdate ion undoubtedly is distorted, we must use crystallochemical and geometrical considerations, if we want to fix the oxygen positions more accurately. Two oxygen atoms O_1 and O_2 may be placed on the middle of the vectors I–III and II–IV, sharing corners; thus $O_1 = (18.8/60, 18.2/120, 13.6/60)$ and $O_2 = (11.3/60, 11.4/120, \overline{2.6}/60)$. Their distances to neighbouring Mo atoms are O_1–I = O_1–III = 2.15 Å, O_2–II = O_2–IV = 2.14 Å, O_1–V = 2.5 Å, O_1–VI = 2.5 Å, O_2–VI = 2.6 Å and O_2–VII = 2.4 Å. These distances are extremely long (cf. 2.0–2.5 Å in (12)), and a displacement of the oxygen atoms nearer to V, VI and VII is probable. Put for instance $O_1 = (20.0/60, 18.2/120, 13.6/60)$ and $O_2 = (13.0/60, 11.6/120, \overline{2.6}/60)$. We then obtain O_1–V = 2.3 Å, O_1–VI = 2.4 Å, O_2–VI = 2.4 Å, O_2–VII = 2.3 Å and O_1–I = 2.1 Å, O_1–III = 2.2 Å, O_2–II = 2.2 Å and O_2–IV = 2.2 Å. It is evident, that we can move the oxygen atoms rather easily to get reasonable Mo–O distances. Such a distribution is, however, of little interest. If we cannot give the oxygen atoms definite places from the experimental data, we cannot obtain any knowledge in addition to the agreement with earlier data on Mo–O distances and with symmetry requirements. The only point of interest in this study is that the only oxygen atoms which are rather definitely fixed by the structure of the ion, viz. O_1 and O_2, easily can be given parameters in agreement with our crystallo-chemical and geometrical assumptions. For the other oxygen atoms this is almost trivial. There is one further fact which must be noticed in this connection. The vectors O_1–V is, after our last displacement, [14.0/60, 4.9/120, 7.6/60] and the vector O_2–VI is [15.5/60, 4.8/120, 7.1/60]; thus their maxima in P(x, y, z) should nearly coincide. But due to the parallelism and the octahedral symmetry of all the MoO_6 octahedra there must be two rather equal Mo–O vectors, giving together 14 nearly identical maxima. Now it happens that the vector O_1–I is [7.0/60, $\overline{5.3}$/120, 8.4/60] and there must also be 14 vectors of this kind. Furthermore P(x, y, z) is identical with P(x, $\bar{y}$, z) so that in P(x, 5/120, z) all these 28 vectors Mo–O vectors must overlap. Just in this region we have the extended and large maximum not explained in any other way. Finally there are 14 Mo–O distances equal to O_1–VI [8.5/60, 1.8/120, 9.$\bar{1}$/60] and to O_2–VII

$[6.0/60, 2.7/120, \overline{9.6}/60]$ which must occur as maxima in P(x, y, z). Here too there is a rather extended maximum of the same height as most Mo–Mo maxima. The principal structure of the $Mo_7O_{24}^{6-}$ ion is thus completely confirmed by P(x, y, z).

Ammonium and water positions. Crystal structure

All discussions in the preceeding parts clearly indicate that there is no hope of determining the positions of the ammonium ions and water molecules directly from the experimental data in this case. A thorough investigation of the mutual orientation of the paramolybdate ions in the crystal combined with space considerations, using some earlier data on the radius of O^{2-} may possibly give electrostatic evidence for suitable places for the ammonium ions. The normal plate form of the crystals may give some further information about the H_2O positions. As the primary problem for us has been the structure of the paramolybdate ion, we have not tried to proceed farther in this direction. We have found, however, that rubidium paramolybdate $Rb_6Mo_7O_{24} \cdot 4\,H_2O$ is isomorphous with $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ and has exactly the same dimensions. As Rb is nearly as heavy as Mo this fact opens new possibilities for determining the positions of the alkali ions, and to that end we have started an investigation of the rubidium salt.

Molybdenum–oxygen coordination in the molybdates

One of the most important aims of this investigation was to obtain new knowledge about the crystal chemistry of the molybdates. In this respect it is very interesting to note the existence of a large condensed anion polyhedron built up by MoO_6 octahedra. Other types of condensed MoO_6 anion polyhedra are the $PMo_{12}O_{40}^{3-}$ ion in the phosphomolybdates (14, 15) and $TeMo_6O_{24}^{6-}$ in the molybdotellurates (13). Both these types of ions, though different from $Mo_7O_{24}^{6-}$ have MoO_6 octahedra as their building units. The MoO_6 octahedra are also found in MoO_3 (11, 12) and the lower oxides of Mo (10). On the other hand, the existence of tetrahedral MoO_4^{2-} ions has been shown in i.a. Ag_2MoO_4 (16), $CaMoO_4$, $BaMoO_4$, $PbMoO_4$ (17), $LiLa(MoO_4)_2$, $Na_5Bi(MoO_4)_4$ (18). The question then arises what factors govern the oxygen coordination of Mo in the molybdates. If we consider the anydrous molybdates at first it seems reasonable to suppose that the ratio Mo:O is the most important factor. In order to study this question further and eventually to determine the limiting Mo:O ratio, we are now investigating the crystal structure of some anhydrous di- and trimolybdates. It is possible that the same factor also governs the coordination in the hydrated molybdates. In this series, however, $(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$ is the only compound which has been investigated. Neither any hydrated normal molybdate, nor any hydrates of MoO_3, the limiting compounds of the series, have been studied. We have therefore also started studies of $Na_2MoO_4 \cdot 2\,H_2O$ and $MoO_3 \cdot 2\,H_2O$. It would, of course, also be valuable to obtain the structures of some hydrated tri- and tetramolybdates, and we hope to be able to study such compounds. Obviously there is not yet sufficient experimental material for any further discussion of these problems.

Chemistry of the paramolybdates

Before discussing the chemistry of the paramolybdates in the light of the results of this investigation, we ought to state clearly the definite new facts established in this study and the conclusions which can be drawn from these facts. The existence of an ion $Mo_7O_{24}^{6-}$ in the solid state and its structure has been established quite definitely (fig. 12 a and b). Thus no water molecules are part of the ion. A most plausible assumption then seems to be that this ion must in some way preexist in water solutions, from which the paramolybdate crystallizes. In the solution the whole ion $Mo_7O_{24}^{6-}$ or fragments of this ion may exist. In any case it may be permitted to assume that there are mainly ions built up by MoO_6 octahedra in the solution. A definite proof will possibly be given by an X-ray diffraction study of the solution, which is planned in this institute.

We have already made comments on three of the four most important problems in the chemistry of the paramolybdates:

1. Formulae of the paramolybdates.
2. Structure of the paramolybdate ion in the solid state.
3. Conditions in water solutions.
4. Relations to other molybdates.

The 3: 7 formulae were definitely confirmed by STURDIVANT (2) and LINDQVIST (3). (Cf. the perparamolybdates (4).) Even before that, however, these correct formulae had been assumed by many authors (cf. the short historical review in (3)). The confusion was due, partly to the small difference in mole ratio between 3: 7 and 5: 12 not easily determinable by analytical methods, partly to erroneous comparisons with the paratungstates. Thus some authors giving the correct 3: 7 formula for the paramolybdates, claimed that the same formula was also correct for the paratungstates, which have a 5: 12 formula (19) and vice versa. As early as 1913, however, COPAUX (20) pointed out the difference between paramolybdates and paratungstates manifested by the absence of isomorphism. In most subsequent studies due regard has not been paid to this work. Especially the uncritical analytical work of ROSENHEIM and his school (21), supported by ROSENHEIM's authority has contributed to the wide acceptance of the wrong formulae of the 5: 12 type.

If we then consider the works on the structure of the paramolybdate ion, the most accepted structure, a coordination structure following WERNER, has been put forward by ROSENHEIM (21). For the reason mentioned above his theories of structures such as $(NH_4)_5H_5\,[H_2(MoO_4)_6]$ have only historical interest. The same is the case with JANDERS hypothetical $(NH_4)_5\,[HMo_6O_{21}]$ (22). COPAUX (20) formulated the ion as $[MoO_3(Mo_2O_7)_3]^{6-}$. There are, however, no signs of Mo_2O_7 groups in the structure. Nor does the formula $[O_3MoO(OMoO_2)_5MoOO_3]^{6-}$ proposed by POSTERNAK (23) correspond to the real structure. As a matter of fact it seems impossible to represent the real structure by a formula in one plane. The structure hypothesis put forth by ANDERSON (24, 25) is worth mentioning in this connection, as this structure recently has been found in the molybdotellurate ion, $TeMo_6O_{24}^{6-}$ (13). ANDERSON assumes a six-membered ring of MoO_6 octahedra sharing edges. A seventh octahedron has room in the middle of the ring. There is a great resemblance between this structure and the structure of $Mo_7O_{24}^{6-}$. If we (in Fig. 12 a) move the octahedra III and IV, so that their centers are placed in the same plane as the centers of I, II, V, VI and VII we obtain ANDERSON's structure. When the seventh atom is of another kind than Mo it seems rather natural that the centrosymmetric ion structure is formed, while the structure of $Mo_7O_{24}^{6-}$ as the most compact one is preferred, if only Mo atoms have to build up the ion. Finally it may be mentioned that a theoretical derivation of a structure of the paramolybdate ion, based on the wrong assumption that Mo-O polyhedra must not share edges has been published by Riesenfeld (26).

Many investigations have been devoted to the study of the water solutions of paramolybdates by different physico-chemical methods, recently reviewed by Carpéni (27). The most important studies have undoubtedly been carried out by cryoscopic methods and by diffusion and dialysis methods. Both these methods indicate the existence of polymolybdate ions containing several Mo atoms in acid solutions, but they have given different results in the details. The cryoscopic investigations have been of three kinds: at the freezing point of water, at the transition point of sodium sulphate (between anhydrous salt and the decahydrate) and at some eutectic points. The works by Travers and Malaprade (28), Darmois and Perin (29), Byé (30, 34) and Doucet (31), all have led to the same conclusion, namely that there are ions containing seven Mo atoms in the solution. The other direct methods for determination of the ion weights, diffusion and dialysis, have been used by Jander (22) and Brintzinger (32), both of whom obtained the same result, namely that the paramolybdate ion in solution has the formula $HMo_6O_{21}\,.\,aq^{5-}$. Their methods have been criticized by Carpéni (27) and Souchay (33), who point out the difficulty arising from the successive disaggregation, surely occuring during diffusion to smaller concentrations, and the impossibility of accounting for the hydration. For a detailed discussion the reader is referred to Souchay (33). Only one further point from his work will be mentioned here. He shows that if the ion weight 900 obtained for $HMo_6O_{21}^{5-}$ with a diffusion coefficient $D = 0.28$ is used as a reference, very confusing results are obtained in determining the ion weights of the polyvanadates. These critical studies indicate that it is not possible to decide between an ion containing six or seven Mo atoms in Janders and Brintzingers measurements. Furthermore the conductometric measurements by Jander (22), supporting his theories, have not been confirmed by Byé (34). If we yet regard the possibilities of getting an ion with six Mo atoms from $Mo_7O_{24}^{6-}$, we notice that the subtraction of the central octahedron VI results in the formation of an ion $Mo_6O_{24}^{12-}$ and the subtraction of any other octahedron in the formation of an ion $Mo_6O_{22}^{8-}$, neither of them in agreement with the theories of Jander. It seems thus most probable that $Mo_7O_{24}^{6-}$ exists also in water solutions. A definite answer could possibly be given by an X-ray diffraction study of the solution. An argument seemingly in favor of an ion containing six Mo atoms was given by Rosenheim (21), who pointed out that paramolybdate solutions easily form 6-heteropolymolybdates with several metal salts solutions. We have, however, already discussed the close resemblance between $Mo_7O_{24}^{6-}$ and $TeMo_6O_{24}^{6-}$, which may easily explain this formation.

Conductometric and potentiometric investigations have not been succesful in explaining the intricate conditions in water solutions or the relation of paramolybdates to other molybdates. Carpéni (27) has, however, definitely concluded that in very dilute solutions there exist only MoO_4^{2-} ions. There is another fact, described by Carpéni (27), which is very interesting. He has shown that by acidifying the molybdates the equilibrium is not instantly attained, the solution giving different titration curves at different times. In this connection reference may be given to the equilibrium study of the system MoO_3–Na_2O–H_2O carried out by Byé (34, 35). Byé states that the paramolybdate is in a metastable equilibrium, the stable compound being dimolybdate, which is very difficult to obtain. A strange experimental contradiction must also be mentioned here. The diffusion experiments, which surely are qualitatively correct, indicate the existence of very large aggregates at the iso-electric point (22). Cryoscopic measurements of molybdic acid solutions, on the other hand, have led to ions or molecules containing only four Mo atoms (31). From acidified molybdate solutions of the type investigated by JANDER (22), however, there never crystallize $MoO_3 . 2\,H_2O$ or $MoO_3 . H_2O$ crystals, but only polymolybdates, most frequently described in the literature as octa- and decamolybdates. Our preliminary investigations on these compounds have shown that their powder photographs are very complicated and rich in lines like those of paramolybdates. The powder photographs of $MoO_3 . 2\,H_2O$ are very simple, even simpler than that of e.g. Na_2MoO_4. Considering all these facts, we may put forward a hypothesis about the relations between the different molybdate ions in the solid state and in water solutions. We assume, that there are two series of molybdate ions, one series beginning with the MoO_4^{2-} ion established in water solution (27), leading over dimolybdate ions to the simple molybdic acid containing four Mo atoms, and another series beginning with the paramolybdate ion, leading over higher polymolybdates to very large aggregates at the isoelectric point. The transformation between these series, may be slow due to great differences in structures of the ion species of the two series.

The paramolybdate ion contains no hydrogen, it is a normal salt hydrate of an hypothetical acid $H_6Mo_7O_{24}$. ROSENHEIM (21) devoted much work to this question and definitely stated that the paramolybdates are acid salts. His conclusions are based on dehydration experiments, measuring the dehydration velocity, and his arguments are no doubt correct when he speaks about "Konstitutionswasser", but his conclusions are wrong when he states that such water must be part of a complex polymolybdate ion (cf. the difficulties arising for ROSENHEIM in explaining the formula of guanidinium paramolybdate, which does not contain sufficient water (21)). The water molecules evidently play an important role in the building up of the whole crystal structure of the paramolybdates, but they need not be parts of the paramolybdate ion, and they are evidently not so. Higher polymolybdates may be acid paramolybdates or at least stand in simple relation to the $Mo_7O_{24}^{6-}$ ion. POSTERNAK (23) claims to have obtained such acid paramolybdates, and it would be interesting to repeat his investigation, using powder photographs for the identification. Finally it must once again be stated, that we have here only given a hypothesis, but as we hope, a working hypothesis which will be useful for the continued work on these problems.

SUMMARY

The definite structure of the paramolybdate ion $Mo_7O_{24}^{6-}$ has been completely derived from an X-ray crystallographic investigation of $(NH_4)_6Mo_7O_{24} . 4\,H_2O$, using three-dimensional Fourier methods. The crystal chemistry of the polymolybdates and their behaviour in water solution has been discussed.

For an unvaluable help in the very time consuming check of the intensities and for further aid in the calculation, I wish to thank Mr BENGT LINDQVIST. The work has been financially supported by a grant, which is here gratefully acknowledged, from Statens Naturvetenskapliga Forskningsråd. Finally I wish to thank professor G. HÄGG for the facilities put at my disposal.

Institute of Chemistry, University of Uppsala, January 1950.

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Muccopolysaccharase activity of sea urchin sperms

By GUNNAR LUNDBLAD and ALBERTO MONROY

With 1 figure in text.

After the fertilization of a sea urchin egg the jelly coat seems to be more easily removed from the egg. HARTMANN and SCHARTAU (1) have claimed that a substance which attacks the jelly coat called "jelly coat dissolving factor" or androgamone II is present in the spermatozoa. MONROY and RUFFO (2) and RUFFO and MONROY (3) prepared extracts from spermatozoa of Arbacia lixula and Sphaerechinus granularis by CLAUDE and DURAN REYNALS's (4) method. These extracts were found to dissolve jelly coat sea urchin eggs in vivo and to lower the viscosity of a mucin solution. The active factor was considered to be a hyaluronidase-like enzyme. This conclusion, however, has been criticised to some extent especially because further investigations failed to detect any hyaluronic acid in jelly coat preparations (5, 6, 7). As a matter of fact the substrate used in the experiments was a crude preparation of mucin from umbilical cord. These writers have therefore decided to the problem using more appropriate substrates. As one of the present writers (G. L.) has recently demonstrated the presence of proteolytic activity in sea urchin sperm (8, 9), it seemed interesting to test the jelly-coat splitting extracts for proteolytic activity too. This may also play a rôle in the fluidification of the jelly coat. In the present investigations potassium hyaluronate[1], jelly coat solutions and gelatin were used as substrates.

The writers wish to publish the investigations in the present somewhat incomplete state, since it is uncertain when they will be able to perform further experimental work.

The experiments were carried out at the Zoological Station of Naples, part I in the spring of 1948 and part II in the spring of 1949.

PART I

Hyaluronate-splitting activity

Material and methods. The spermatozoa from Paracentrotus lividus and Arbacia lixula were lyophilized and the extracts prepared by Madinaveitia's method (10, 11), i.e. the sperm material (0.1 per cent dry weight) was rubbed with sand in M/10 acetic acid, the centrifuged extracts were precipitated with ammonium sulphate of between 27 and 70 per cent saturation and the precipitate dialysed against

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[1] We are indebted to the Glaxo Corp. for supplying a sample of pure K-hyaluronate.

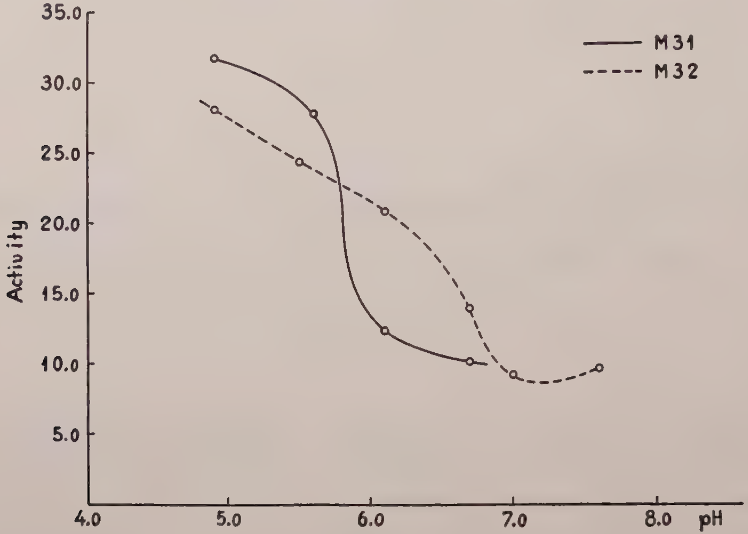

Fig. 1.

sea water or 3.6 per cent sodium chloride. 0.1 per cent potassium hyaluronate dissolved in MacIlvain buffer was used as substrate. The enzymic activity was determined viscosimetrically at 25° C and calculated according to Hultin's formula (9, 12), in which the slope of the line formed by plotting the inverse values of the specific viscosity against time is multiplied by the square of the concentration of the substrate.

Results. Fig. 1 shows the hyaluronate activity at different pH:s for two Paracentrotus extracts treated in different ways. M 31 was dialysed against sea water and M 32 against 3.6 per cent sodium chloride. Both curves show decreasing activity towards neutral pH and both seem to attain the same value there. At pH 5, M 31 is more active due perhaps to the presence of calcium ions in the sea water. WERLE, TURTUR and BAUEREIS (13) found that Ca^{++} increased the activity of mucopolysaccharase from bull testis.

It seems that sea urchin sperm extracts do possess a hyaluronate-splitting activity. This is, however, rather weak, especially as compared with the tremendous activity of preparations from mamalian sperm. Since, as mentioned above, no hyaluronic acid has been found in the jelly coat, the real nature of this splitting activity remains questionable. In view of the results which will be dealt with in the next part, which show the presence in sea urchin sperm extracts of a jelly-coat splitting activity, the reported effect might perhaps be considered as a rather unspecific mucopolysaccharase activity.

PART II

Jelly-coat splitting activity

Material and methods. The sperm material was from Arbacia lixula and Paracentrotus lividus. The preparation of the different extracts is given in Table 1. The jelly-coat solutions were prepared by dissolving the jelly coats from unfertilized

Table 1

Preparations of the Sperm Extracts.

All extracts were made at + 2° C. Sea-w. = sea water. pH of extracts 7.5.

Extract	Material		Extract		Extraction time	Dialysis medium	N in mg/ml	Note
	Species	Amount	ml	kind				
Asp 1	Arbacia lyophilized	0.5 g	20	M/10 HAc	7 hours	Sea-w.	0.10	
Psp 2	Paracentrotus lyophil.	0.5 »	20	» »	7 »	»	—	
Asp 30	Arbacia frozen	—	—	Sea-w.	5 days	»	0.30	To the frozen sperm was added 3 times its volume of sea-water
Asp 31	»	—	—	M/10 HAc	5 »	«	0.08	»
Asp 32	»	—	—	» »	10 »	»	0.09	»
Psp 31	Paracentr. frozen	15 ml	15	» »	6 »	»	1.78	
Asp 41	Arbacia, frozen	20 »	20	» »	7 »	»	—	
Asp 42	»	20 »	20	» »	20 »	Dist. w.	0.75	After dialysis the extract was lyophilized and the dry material dissolved in 3.5 ml sea water (N = 0.075 %)
Asp 51	»	40 »	40	Sea-w.	5 »	»	4.98	

Arbacia and Paracentrotus eggs in acidified sea water (pH 5.5). After centrifuging the jelly-coat substance was precipitated with ethanol and redissolved by dialysis against running tap water followed by dialysis against sea water. The activity was determined viscosimetrically at 26.3° C and calculated as in Part I.

Results. The results of the activity measurements are shown in Table 2. We find from experiments 1–4 that an acetic acid extract of Arbacia possesses a viscosity lowering action on Arbacia jelly coat which is about 2.5 times higher than in the boiled control. We also find that it has no effect on Paracentrotus jelly coat. In experiment 16–18 it is of interest to see that 10 minutes boiling does not alter the activity of the extract, but that 40 minutes boiling does. That a Paracentrotus extract has a splittering effect on Paracentrotus jelly-coat solution is shown by experiments 29–31, and that a Paracentrotus extract has none on an Arbacia jelly-coat solution is shown by experiments 6 and 7. The Arbacia extract Asp 1 is also active here. Thus it may be concluded from this investigation that both Arbacia and Paracentrotus sperm extracts have a splittering effect on jelly-coat solutions from their own species and also that Arbacia has no "cross" effect on Paracentrotus. In the case of the Paracentrotus effect on Arbacia, however, we cannot be sure because we used different extracts in experiments 6 and 29. The influence of boiling for different times on the activity of the extracts appears to be very clear in exper-

Table 2

Jelly Coat Splitting Activity of Sperm Extracts.

The reaction mixture was made up with 3.00 ml. substrate and 0.50 ml. boiled or unboiled extract. The activity cannot be noted in Hultin Units as the concentration of the substrate is unknown.

Exp. No.	Extract	N in mg/ml	Substrate: Jelly coat	Treatment of extract	Comparative Activity	
					per ml	per g N × 10^4
1	Asp 1	0.10	Arbacia	—	11.67	11.67
2	»	0.10	»	Boiled 10′	4.67	4.67
3	Asp 1	0.10	Paracentr.	—	0	0
4	»	0.10	»	Boiled 10′	0	0
6	Psp 2	—	Arbacia	—	0	0
7	Asp 1	0.10	»	—	21.40	21.40
12	Asp 31	0.08	Arbacia	Boiled 10′	3.79	4.75
13	»	0.08	»	—	5.83	7.29
14	Asp 30	0.30	Arbacia	—	4.08	1.36
15	»	0.30	»	Boiled 10′	10.50	3.50
16	Asp 32	0.09	Arbacia	—	2.33	2.59
17	»	0.09	»	Boiled 10′	2.33	2.59
18	»	0.09	»	» 40′	1.17	1.30
19	Asp 32	0.09	Paracentr.	—	0	0
20	»	0.09	»	Boiled 40′	0	0
26	Asp 41	—	Arbacia	—	0	0
27	»	—	»	Boiled 10′	0	0
29	Psp 31	1.78	Paracentr.	—	7.58	0.427
30	»	1.78	»	Boiled 5′	4.67	0.262
31	»	1.78	»	» 30′	1.75	0.098
35	Asp 42	0.75	Arbacia	—	9.33	1.24
36	»	0.75	»	Boiled 5′	6.42	0.856
37	»	0.75	»	» 25′	3.50	0.467

iments 29–31 and 35–37. In Asp 30, however, the effect was greater after 10 minutes boiling (cf Table 3, Exp. 23).

The proteolytic activity of some of these sperm extracts is shown in Table 3, Asp 30, which was extracted with sea water for 5 days, has an extremely high activity as compared with Asp 32 which is an acetic acid extract from the same batch. That water extract possesses higher activity calculated on ml solution than acetic acid extracts has been shown before by one of us (9). The still higher activity in experiment 34, where Arbacia jelly-coat solution is added to the sea water extract Asp 51, is probably due to the proteolytic activity of the jelly-coat solution, also reported earlier (9).

It seems possible, although not definitely established, that the hyaluronidate and jelly-coat splitting activity is due to the same enzyme. On the other hand it seems unlikely that this enzyme would also be responsible for the proteolytic activity. One of us (14) found that sperm extracts from stored dried material posses-

Table 3

Proteolytic Activity of the Sperm Extracts.

Substrate: 4.0 % gelatin. pH 7.8. Activity in Hultin Units. Temperature 35.5° C.

Exp. No.	Extract	N in mg/ml	Reaction mixture: volume in mm	Activity		Note
				A 10^9/ml	A 10^6/g N	
21	Asp 32	0.09	0.50 Asp 32+3.00 Gel.	20.6	229	
23	Asp 30	0.30	0.50 Asp 30+3.00 Gel.	400.0	1 340	
34	Asp 51	4.98	(0.50 Asp 51+0.50 Arb Jc)+2.50 Gel.	631.0	31.6	This mixture formed a precipitate wich was centrifuged off. To the clear solution was then added 2.50 ml gelatin
38	Asp 42	0.75	0.50 Asp 42+3.00 Gel.	180	240	

sing proteolytic activity showed no hyaluronidate-splitting activity. This indicates the presence of two separate enzymes of which the hyaluronidate-splitting is less resistant to storage or to the freeze drying process. The possibility must be considered, however, that the splitting of the jelly-coat substance is due to proteolytic activity, which would affect the protein links within the jelly.

SUMMARY

Hyaluronidate-splitting activity is found in acetic acid extracts from Paracentrotus sperms. The activity depends within a certain pH-interval on the dialysis medium of the extract.

Extracts from Arbacia and Paracentrotus sperms have a viscosity lowering effect on jelly-coat solutions of their own species. It is shown that Arbacia extract has no activity on Paracentrotus jelly coat and that the reverse is possibly also the case. The extracts have a strong proteolytic activity.

The writers wish to thank Prof. J. Runnström for his active interest in our work and Dr T. Hultin and Dr E. Vasseur for their suggestions.

From the Wenner-Gren Institute for exp. Biology, University, Stockholm, and the Zoological Station, Naples.

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Communicated 10 May 1950 by A. WESTGREN and G. HÄGG

The structure of the tetramolybdate ion

By INGVAR LINDQVIST

With 2 figures in the text

In a preliminary communication (1) a new theory of the nature of the tetramolybdate ion was put forward. The meta- and hexamolybdate theories (2, 3) have been ruled out and it has been proposed that the tetramolybdates are built up by octamolybdate ions. This proposal has now been proved to be correct by a crystal structure investigation of $(NH_4)_2O \cdot 4\,MoO_3 \cdot 2.5\,H_2O$.

Crystals

A prismatic crystal (0.5 mm thick) was used for the investigation. This thickness will give rise to considerable absorption errors in the intensities, but we could not find a thinner crystal. Using Cu–K radiation, rotation and Weissenberg photographs were taken around the c-axis of all available layer lines ($l = 0 - 5$). The intensities were visually estimated and corrected according to LU (4). The crystal system was found to be triclinic with the unit cell dimensions $a = 7.76$ Å, $b = 9.75$ Å, $c = 9.78$ Å, $\alpha = 97^\circ.2$, $\beta = 100^\circ.5$ and $\gamma = 98^\circ.4$. (The axes are chosen in agreement with the rules given by BUERGER (5)). As the crystals show no sign of piezo-electricity when investigated by a dynamic method the space-group may be $C_i^1 - P\,\bar{1}$.

Molybdenum positions

There are eight Mo atoms in the unit cell (1), the parameters of which must be determined. Instead of trying to interpret a Patterson-projection p(x, y), a three-dimensional Patterson synthesis was carried out. P(x, y, z) were calculated for x = 0 — 30/30, y = 0 — 15/30 and z = 0 — 30/30. The maxima obtained could be divided into groups with different heights. The three largest maxima, denoted A, B, and C are situated at:

A: x = 0.83, y = 0.02, z = 0.28

B: x = 0.85, y = 0.20, z = 0.76

C: x = 0.15, y = 0.33, z = 0.08.

The next six maxima a–f, approximately half as high as A–C, have the coordinates:

a: x = 0.30, y = 0.13, z = 0.32
b: x = 0.02, y = 0.18, z = 0.47
c: x = 0.69, y = 0.21, z = 0.04
d: x = 0.32, y = 0.32, z = 0.80
e: x = 0.98, y = 0.34, z = 0.36
f: x = 0.01, y = 0.47, z = 0.17

We may now discuss what arrangement of eight Mo atoms is consistent with these vectors. The assumption of an octamolybdate ion with centro-symmetry gives an atomic configuration corresponding to the corners of some parallelepipedon (Fig. 1), which has three edges, each occurring four times, six face-diagonals, each occurring two times, and three space-diagonals. As we have found groups of three and six vectors it remains to be seen whether they fulfil the necessary relations. We easily find that

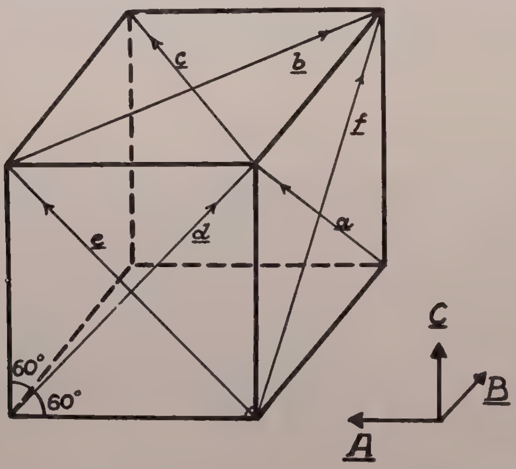

Fig. 1. The parallelepipedon corresponding to the eight Mo atoms.

$$\begin{cases} B + A = c, \\ B - A = b, \end{cases} \quad \begin{cases} C + A = e, \\ C - A = d, \end{cases} \quad \begin{cases} C + B = f \\ C - B = a. \end{cases}$$

These vector equations mean that c and d are diagonals in a parallelogram formed by A and B and so on (Fig. 1). As a further check, the space-diagonal vectors were calculated and the maxima were found at the correct positions in P (x, y, z), and with half the height of a–f:

$$\text{I: } x = 0.17,\ y = 0.45,\ z = 0.88$$
$$\text{II: } x = 0.83,\ y = 0.49,\ z = 0.44$$
$$\text{III: } x = 0.13,\ y = 0.15,\ z = 0.60$$
$$\text{IV: } x = 0.47,\ y = 0.11,\ z = 0.04.$$

These vectors evidently correspond to distances between (x, y, z) and $(\bar{x}, \bar{y}, \bar{z})$, and thus determine the parameters of the Mo atoms. If the parameters are chosen in agreement with the other vectors, one proper parameter set of the six identical ones is the following:

$$\text{Mo}_\text{I}\ : x = 0.42,\ y = 0.27,\ z = 0.55$$
$$\text{Mo}_\text{II}\ : x = 0.27,\ y = 0.95,\ z = 0.48$$
$$\text{Mo}_\text{III}: x = 0.57,\ y = 0.07,\ z = 0.80$$
$$\text{Mo}_\text{IV}: x = 0.58,\ y = 0.25,\ z = 0.78.$$

As the intensities are known only very approximately (v. supra), a comparison between calculated and observed intensities cannot be at all conclusive. A calculation of $|F(h\,k\,0)|$ has, however, been carried out. (Table 1. All values are reduced to an intensity scale 0—3 to show the crude but clear agreement.) It may finally be noted, that in this case the configuration of the Mo atoms and even the structure of the tetramolybdate ion (v. infra) could be completely determined without any knowledge about the crystallographic parameters of the Mo atoms, which only give the orientation of the ion in the unit cell.

Oxygen coordination

It is possible in this case, just as for the paramolybdate ion (6) to derive the oxygen coordination from crystal chemical considerations. First we calculate the lengths of the vectors A, B, and C, which are 3.25, 3.20 and 3.22 Å. In the paramolybdate ion, $Mo_7O_{24}^{6-}$, Mo—Mo distances corresponding to MoO_6 octahedra sharing edges have values between 3.21 and 3.48 Å. Thus it can be definitely stated that A, B, and C join centers of MoO_6 octahedra sharing edges. A study of the vectors a—f indicates that d and e are both 4.5 Å, which is $3.2\sqrt{2}$ Å. A and C thus form a square of octahedra sharing edges. The ions must be built up by two such squares and it remains to be determined how they are coupled together. As a and c are 3.51 and 3.43 Å, there must be further sharing of edges in two other directions, and we then arrive at the structure illustrated in Fig. 2 a and b. All vectors required by this structure are found in P (x, y, z). Evidently one of the space-diagonals must also correspond to edge sharing and accordingly its value is 3.56 Å. The ion is obviously built up of rather regular squares of octahedra, but these squares are coupled together with small distortions (compare 3.25, 3.20, 3.22 Å with 3.51, 3.43 and 3.56 Å). The formula of this ion is $Mo_8O_{26}^{4-}$ giving the formula $(NH_4)_4Mo_8O_{26}\cdot$ $\cdot\, 5\,H_2O$ of the salt investigated. As to the parameters of the oxygen atoms cf.

Table 1

Comparison between calculated and observed $\lvert F \rvert$-values.

h k 0	$\lvert F \rvert$		*h k* 0	$\lvert F \rvert$		*h k* 0	$\lvert F \rvert$	
	calc.	obs.		calc.	obs.		calc.	obs.
1 0 0	3	3	2 3 0	0	0	$\bar{4}$ 5 0	0	0
2 0 0	1	1	3 3 0	0	0	$\bar{5}$ 5 0	0	0
3 0 0	0	0	4 3 0	0	0	0 6 0	2	3
4 0 0	0	0	5 3 0	1	1	1 6 0	1	1
5 0 0	1	1	$\bar{1}$ 3 0	0	0	2 6 0	0	0
6 0 0	2	2	$\bar{2}$ 3 0	1	1	3 6 0	0	0
7 0 0	2	2	$\bar{3}$ 3 0	0	0	4 6 0	0	0
0 1 0	2	2	$\bar{4}$ 3 0	0	0	5 6 0	2	2
1 1 0	1	2	$\bar{5}$ 3 0	0	0	6 6 0	2	2
2 1 0	0	0	$\bar{6}$ 3 0	1	1	$\bar{1}$ 6 0	2	3
3 1 0	0	0	0 4 0	2	2	$\bar{2}$ 6 0	1	1
4 1 0	1	1	1 4 0	2	2	$\bar{3}$ 6 0	0	0
5 1 0	0	0	2 4 0	1	1	$\bar{4}$ 6 0	0	0
6 1 0	0	0	3 4 0	0	0	$\bar{5}$ 6 0	0	0
$\bar{1}$ 1 0	0	0	4 4 0	0	0	0 7 0	0	0
$\bar{2}$ 1 0	1	1	5 4 0	0	0	1 7 0	0	0
$\bar{3}$ 1 0	0	0	$\bar{1}$ 4 0	0	0	2 7 0	0	0
$\bar{4}$ 1 0	0	0	$\bar{2}$ 4 0	0	0	3 7 0	0	0
$\bar{5}$ 1 0	0	0	$\bar{3}$ 4 0	0	0	4 7 0	1	1
$\bar{6}$ 1 0	1	0	$\bar{4}$ 4 0	0	0	5 7 0	0	0
0 2 0	1	1	$\bar{5}$ 4 0	2	2	$\bar{1}$ 7 0	0	0
1 2 0	0	0	$\bar{6}$ 4 0	2	1	$\bar{2}$ 7 0	0	0
2 2 0	1	1	0 5 0	1	2	$\bar{3}$ 7 0	1	0
3 2 0	0	0	1 5 0	0	0	0 8 0	0	0
4 2 0	2	2	2 5 0	0	0	1 8 0	1	0
5 2 0	2	2	3 5 0	0	0	2 8 0	0	0
$\bar{1}$ 2 0	2	3	4 5 0	1	0	3 8 0	1	1
$\bar{2}$ 2 0	2	2	5 5 0	2	2	4 8 0	1	2
$\bar{3}$ 2 0	0	0	6 5 0	1	1	$\bar{1}$ 8 0	1	0
$\bar{4}$ 2 0	0	0	$\bar{1}$ 5 0	2	2	$\bar{2}$ 8 0	1	1
$\bar{5}$ 2 0	0	0	$\bar{2}$ 5 0	1	0	$\bar{3}$ 8 0	1	0
0 3 0	1	1	$\bar{3}$ 5 0	0	0	0 9 0	2	2
1 3 0	2	3						

the more complete discussion of the paramolybdate ion (6). The close resemblance with the paramolybdate ion $Mo_7O_{24}^{6-}$ is easily seen from Fig. 2 b and d. Six of the MoO_6-octahedra are attached in the same way to each other in the two ions. This common part of the two ions corresponds to the formula $Mo_6O_{22}^{8-}$ and is shown in Fig 2 c. Thus just as the groups of 2 + 3 + 2 octahedra give the most compact $Mo_7O_{24}^{6-}$ ion, we here have arrived at the most symmetrical and compact ion $Mo_8O_{26}^{4-}$ with groups of 4 + 4 octahedra.

We have not attempted to determine the ammonium and water positions.

Chemistry of the tetramolybdates

A review of older theories has been given in the preliminary report (1). Here we will consider only the conclusions concerning the solid tetramolybdates and

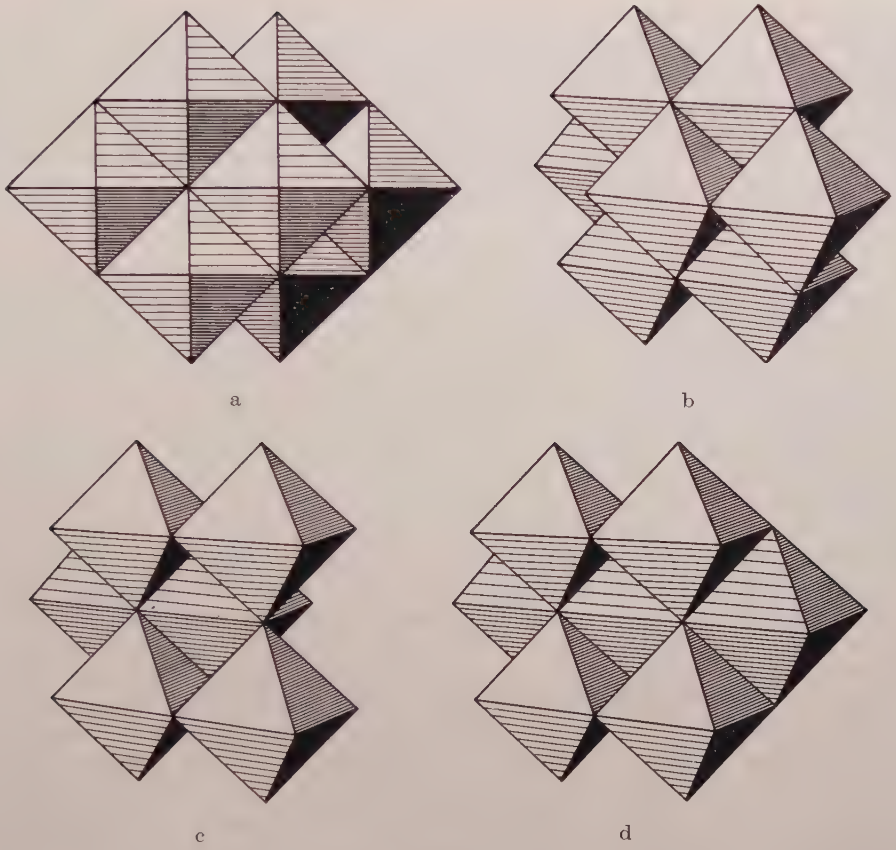

Fig. 2 a and b. The tetramolybdate-ion $Mo_8O_{26}^{4-}$. c. The part common to the ions $Mo_8O_{26}^{4-}$ and $Mo_7O_{24}^{6-}$ and corresponding to the composition $Mo_6O_{22}^{8-}$. d. The paramolybdate ion $Mo_7O_{24}^{6-}$. All the figures are idealized, showing regular MoO_6 octahedra.

their water solutions which can be drawn by a consideration from the new point of view of the structure of the ion $Mo_8O_{26}^{4-}$. The solid tetramolybdates must be written $R_4Mo_8O_{26} \cdot nH_2O$, and are normal salts of an hypothetical acid $H_4Mo_8O_{26}$ rather than acid salts, as has been stated by Rosenheim (2). As this is also the case with the paramolybdates $R_6Mo_7O_{24} \cdot nH_2O$ it seems very probable that the trimolybdates, which crystallize from solutions with pH values in the narrow pH range between the paramolybdate and tetramolybdate solutions, must be normal salts, too.

A fact of interest for the crystal chemistry is of course the resemblance between tetramolybdate and paramolybdate ions, and the obviously primary importance of MoO_6 octahedra sharing edges, in building up the polymolybdate ions.

The ionic state of water solutions of tetramolybdates can be treated very similarly to that of the paramolybdate case to which reference is made. For the tetramolybdate case the diffusion measurements have been explained by Jander (3) by the assumption of an hexamolybdate ion $H_3Mo_6O_{21}$ aq^{3-} (cf.

HMo_6O_{21} aq^{5-} for the paramolybdate ion). The molecular weights being only approximately correct, however, the existence of an octamolybdate ion $Mo_8O_{26}^{4-}$ is not contradicted by these investigations. The close resemblance with the $Mo_7O_{24}^{6-}$ ion permits a direct analogy with the structural discussion of that ion, which in this case leads to the conclusion that it is very probable that $Mo_8O_{26}^{4-}$ ions exist also in water solutions. The only experimental objection against this opinion are the cryoscopic data giving $Mo_4O_{13}^{2-}$, but TRAVERS and MALAPRADE (8), have obtained this result by the assumption of an incomplete dissociation, and BYÉ (10) has only studied very dilute solutions.

It may be mentioned in this connection that the conductometric measurements by JANDER (3) may support the existence of ions $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$ as well as the ions HMo_6O_{21} aq^{5-} and $H_3Mo_6O_{21}$ aq^{3-} proposed by him. He has found breaks in the conductivity curve, when 7 H^+ and 9 H^+ has been added to 6 MoO_4^{2-}, while $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$ require breaks at 6.9 H^+ and 9 H^+.

Considering all known facts we have been led to the assumption that by acidification of water solutions of molybdates the ions $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$ are formed successively in the pH range 4.5—1.5 (cf. CARPÉNI (7)). From structural considerations it seems very improbable that there exist any other types of complex ions with a composition between these two ions. The only molybdates described as being obtained in this region are the trimolybdates $R_2O \cdot 3\,MoO_3 \cdot nH_2O$. The net composition of a normal trimolybdate ion will correspond to $Mo_3O_{10}^{2-}$ but only ions two or three times as large, that is $Mo_6O_{20}^{4-}$ or $Mo_9O_{30}^{6-}$ can be considered. None of these ions has a close structural resemblance with $Mo_7O_{24}^{6-}$ and $Mo_8O_{26}^{4-}$, in which case their formulae should have been $Mo_6O_{22}^{8-}$ or $Mo_9O_{28}^{2-}$. It is therefore improbable that any trimolybdate ions exist. The existence of solid trimolybdates can, however, be explained in another way, viz. considering them as double salts of paramolybdates and tetramolybdates, $R_{10}(Mo_7O_{24})(Mo_8O_{26}) \cdot nH_2O$. This double salt formation may be possible because of the resemblance between the two anions. This is of course only an hypothesis.

Finally it can be mentioned that the working hypothesis of the existence of two series of molybdate ions, put forward in an earlier paper, has not been supported by this study or by that of molybdic acid (9), $MoO_3 \cdot 2\,H_2O$.

SUMMARY

The structure of the tetramolybdate ion $Mo_8O_{26}^{4-}$ has been determined from an X-ray crystallographic investigation of $(NH_4)_4Mo_8O_{26} \cdot 5\,H_2O$. The conditions in water solutions have been discussed and a theory of the nature of the trimolybdates has been put forward.

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Institute of Chemistry, University of Uppsala, May 1950.

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Acetic acid metabolism in Rhodospirillum rubrum under anaerobic condition

By C. CUTINELLI, G. EHRENSVÄRD and L. REIO

During the last years acetic acid has been shown to occupy a central position in the carbohydrate and the fat metabolism of several biological systems. The use of isotope labelled acetate has greatly faciliated the study of the different pathways, over which acetic acid is transformed into lipids and carbohydrate (1), and also the mechanism of its aerobic degradation (2). As to the rôle of acetate in formation of different amino acids some attempts have been made to investigate this problem by the administration of labelled acetate to diverse organisms as the single carbon substrate, isolation of the amino acids and chemical degradation of the latter in order to localize the isotope excess in different carbon atoms of the amino acids. Some experiments of this kind have been carried out with the yeast Torulopsis utilis (3, 4, 5), and in the bacterial series, with Escherichia Coli (6), using acetic acid, labelled with C^{13} in the methyl group, C^{14} in the carboxyl group. The overall results of these experiments show that both isotopes become to a considerable extent incorporated into the proteins, even after a so short period of exposure to the labelled acetate as 2.5 and 3.5 hours respectively.

A feature of special interest in these experiments is that the methyl group of acetate, as indicated by the C^{13} content of the proteins formed, seems to contribute to the carbon skeleton of the amino acids to a higher extent than does the carboxyl group, the ratio C^{13} to C^{14} in the overall proteins being about 2, compared with the corresponding C^{13} to C^{14} ratio in the acetate used, taken as 1. On the other hand the corresponding ratio on the respiratory CO_2 was about 0.5–0.7 indicating that in the aerobic breakdown of acetate more carboxyl groups are entering the decarboxylation cycle than methyl groups.

In view of these results it would be of interest to study the anaerobic utilization of acetate, using double labelled acetate as substrate for a suitable organism. It has been found by van Niel that Rhodospirillum rubrum grows well in an acetate medium under anaerobic conditions under exposure to light, provides that a certain amount of bicarbonate is present, and in addition a small amount of yeast extract, possibly containing some necessary growth factors. The extensive experiments of VAN NIEL (7), definitely indicate that the bicarbonate is not to be considered as a mere buffer component, but that it really functions as one of the carbon sources of the medium. We are thus faced with the necessity of labelling three carbon atoms of the system, the methyl group and the carboxyl group of acetate, and the bicarbonate, the small amount of yeast extract in the medium being regarded as unsignificant as a carbon source for growth.

In the experiment to be described we prepared two series of chemically identically media, one containing $C^{13}H_3C^{14}OONa$ and $NaHCO_3$, the other containing CH_3COONa and $NaHC^{14}O_3$, both of which were inoculated with the same amount of a four days old culture of Rhodospirillum rubrum, growing on the same, but non-labelled medium. The labelled acetate was prepared according to Baddiley, Ehrensvärd et al. (4). The C^{14} containing bicarbonate was made by saturating 1 ml of N. NaOH with C^{14}-containing CO_2, and mixed with a solution of 2 g of sodium-bicarbonate in 100 ml water.

The two series of sterile media, contained each 3 g of sodium acetate (H_2O-free), 2 g of sodium bicarbonate, 2 g of potassium bicarbonate, 1 g of ammonium chloride, 0.2 g of magnesium sulphate, 0.038 g of calcium chloride, 4.5 ml 6 N. hydrochloric acid and 0.5 g of yeast extract (Difco) in one litre of tap water. The inoculum was the same for both series. For this purpose we divided into equal parts 200 ml of a four days old culture, centrifuged bacteria in two bottles of 1.2 litres, one of which contained 1 litre of the $C^{13}H_3C^{14}OONa$ and $NaHCO_3$-medium, the other 1 litre of the CH_3COONa and $NaHC^{14}O_3$-medium. The dry weight of the inoculum was 105.5 mg for each bottle. After inoculation the bottles were saturated with sterile nitrogen, and tightly closed and kept a light cabinet at a temperature of 30° under the continuous illumination with three 100 watt electric bulbs. At 48 hours from the start of the experiment a 100 ml sample was taken from each culture, at 96 hours 200 ml samples were taken and finally at 144 hours the experiment was ended by centrifuging off the bacteria from each culture, washing twice in the centrifuge with 0.9 % sodium chloride solution, then three times with absolute alcohol and three times with ether. The 48 and 96 hour samples were treated the same way. The green alcohol and the red ether extracts from each sample and the residual culture containing the total amount of pigments, were combined *inter se* and evaporated to dryness, so that for each series three different samples of dried pigment were obtained. The extracted bacterias, containing the total bacterial proteins, were obtained as a dry, colourless powder. A small amount of each sample of dry bacteria were extracted at room temperature with 1 N. NaOH, centrifuged, the clear solution precipitated with 20 % trichloroacetic acid solution, centrifuged, washed with water three times, then with alcohol three times and water three times. Comparison of these trichloroacetic acid precipitates with the total dry material of the extracted bacteria would give some information as to the isotope content of the proteins and the total constituents of the bacteria.

The samples of dry bacteria, the proteins and the pigments were all combusted in oxygen with platinum catalyst to CO_2, trapped as barium carbonate, which, after washing and drying was used for the subsequent C^{13} and C^{14} determinations. From each of the media, samples and residual, CO_2 was driven off by adding strong sulphuric acid and passing nitrogen through the solution. The CO_2 was trapped in baryta solution and isolated as barium carbonate.

The determination of the isotope content of the different barium carbonate samples was made, for C^{13}, with aid of a mass-spectrometer, for C^{14} with a standard Geiger counter arrangement. The C^{13}-content was expressed in atom per cent excess, the C^{14}-content in counts per minute per 15 mg of barium carbonate under standard condition. The following table shows the result of the experiment.

As to the growth of the bacteria during the period of the experiment it was found from dry weight estimations that the amount of inoculum (105.5 mg in one litre) was increased to about 960 mg/litre after 48 hours and remained constant (round

Table I

Isotope content of substrate for two series of experiments and the isolated fractions from bacteria during different periods of growth. All fractions are measured as total carbon, isolated as barium carbonate. C^{13} is given as atom per cent excess, C^{14} as counts per minute per 15 mg of barium carbonate.

Time of the experiment in hours.

	0		48		96		144	
	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}	C^{13}	C^{14}
Substrate I								
$C^{13}H_3C^{14}OONa$	3.34	1810						
$NaHC^0O_3$	—	—						
Substrate II								
$C^0H_3C^0OONa$	—	—						
$NaHC^{14}O_3$	—	1660						
Carbonate carbon								
in medium I	0	0	0.28	216	0.34	327	0.31	312
in medium II	—	1660	—	825	—	770	—	728
Pigmentfree dry bacteria I	0	0	1.42	382	1.51	409	1.40	443
II	—	0	—	141	—	165	—	179
TCA-protein I	0	0	1.21	286	1.34	406		
II	—	0	—	146	—	203		
Total pigment I	0	0	1.67	383	1.48	482	1.69	585
II	—	0	—	100	—	106	—	132

1 000 mg/litre) during the next 96 hours. Thus the main period of growth had taken place during the first 48 hours, which in turn is reflected in the isotope values at 48, 96 and 144 hours. These show that the C^{13} and C^{14}-content of the pigmentfree bacteria, the TCA-protein and the pigments is increasing at a steady, but slow, rate between 48 and 144 hours and that the 48-hour fractions already have taken up a very considerable amount of isotope from the media.

A comparison between the values for carbonate carbon of the two differently labelled media shows that in medium I the C^{13} and C^{14} values gradually increase from the value = 0 at the start, reflecting a steady breakdown of the labelled acetate to CO_2. Calculating the percentage of carbonate carbon, which has originated from the methyl group of acetate (C^{13}) and the carboxyl (C^{14}) we arrive to the following values:

	Carbon from CH_3	Carbon from COOH	Ratio $COOH/CH_3$
48 hours	8.3 %	11.9 %	1.44
96 »	10.2 %	18.2 %	1.79
144 »	9.3 %	17.2 %	1.85

This implies that in the gradual and stepwise anaerobic degradation of acetic acid more carboxyl groups are liberated to the medium as CO_2 than methyl groups as shown by the ratios 1.44–1.85. Accordingly relatively more methyl groups must have made up the material of the bacteria. Calculating the percentage of carbon in the bacterial fractions derived from the methyl group and the carboxyl group of acetate (series I) and the carbon taken up from the bicarbonate of the medium (series II) we get the following picture:

	Carbon from CH_3	Carbon from COOH	Carbon from bicarbonate
Pigmentfree dry bacteria			
48 hours	42.5 %	21.1 %	8.5 %
96 »	45.5 %	22.6 %	10.0 %
144 »	42.0 %	24.4 %	10.8 %
TCA-protein			
48 hours	36.2 %	15.8 %	8.8 %
96 »	40.1 %	22.4 %	12.2 %
144 »			
Total pigment			
48 hours	50.0 %	21.1 %	6.0 %
96 »	(44.0 %)	26.6 %	6.3 %
144 »	50.6 %	32.3 %	8.0 %

In the case of pigmentfree bacteria and the TCA-protein isolated it seems that the carbon has been derived from the methyl group of acetate, the carboxyl group and the bicarbonate of the medium in a ratio, approximately 4 : 2 : 1. The pigments have a slightly different ratio. In the case of the strictly anaerobic *Rhodospirillum* the general picture of carbon utilization from acetate seems to be comparable with the same overall acetate utilization in the aerobic *Torulopsis* (3, 4, 5) and *E. Coli* (6) with the important difference that in *Rhodospirillum* a considerable additional amount of carbon is taken up from the bicarbonate pool of the medium (about 10 %). Since about 20 % of carbon in the pigmentfree bacteria and the TCA-protein and about 10 % of carbon in the pigments cannot be accounted for as being derived from either acetate or bicarbonate this deficit must have come from the only remaining carbon source, the yeast extract, which thus seems to function not only as a source of growth factors but in reality is utilized as a carbon source.

It would be of great interest to isolate the different amino acids of the two series to localize, with chemical degradation methods the isotope content of each carbon atoms, in order to compare these values with the corresponding values from Torulopsis and Coli. We have therefor made a large scale culture of *Rhodospirillum* in two series, one on $C^{13}H_3C^{14}OONa + NaHCO_3$ and $CH_3COONa + NaHC^{14}O_3$ yielding about 18 g of dry, pigmentfree bacteria in each series. We hope in the near future to publish the result of this investigation.

SUMMARY

Rhodospirillum rubrum has been cultivated under anaerobical condition on two chemically identical, but differently labelled media, one containing $C^{13}H_3C^{14}OONa + NaHCO_3$, the other $CH_3COONa + NaHC^{14}O_3$. By estimation of the C^{13} and the C^{14} values of the pigmentfree, dry bacteria, the trichloroacetic acid precipitable proteins and the alcohol and ether soluble pigments it has been possible to calculate the

ratio of the utilization of the different carbon sources of the medium. About 45 % of the total carbon of the bacterial proteins is derived from the methyl group of acetate, about 23 % from the acetate carboxyl group and 10 % from the bicarbonate pool of the medium.

From Wenner-Gren Institute for exp. Biology, University of Stockholm.

LITTERATURE. (1) For general discussion of this problem see **K. Bloch,** Cold Spring Harbor Symposia, XIII, 29 (1948) and **H. G. Wood,** ibid. 201 (1948). — (2) **N. Lifson, V. Lorber, W. Sakami** and **H. G. Wood,** J. Biol. Chem. *176*, 1263 (1948). — (3) **G. Ehrensvärd, E. Sperber, E. Saluste, L. Reio** and **R. Stjernholm,** J. Biol. Chem. *169*, 759 (1947). — (4) **J. Baddiley, G. Ehrensvärd, R. Johansson, L. Reio, E. Saluste** and **R. Stjernholm,** J. Biol. Chem. *183*, 771 (1950). — (5) **J. Baddiley, G. Ehrensvärd, E. Klein, L. Reio** and **E. Saluste,** J. Biol. Chem. *183*, 777 (1950). — (6) **C. Cutinelli, G. Ehrensvärd, L. Reio** and **E. Saluste,** unpublished. — (7) **C. B. van Niel,** Bacteriol. Review *8*, 1 (1944).

Tryckt den 22 juni 1950.

Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 22

Communicated 12 April 1950 by JOHN RUNNSTRÖM

The amino-acid metabolism of the developing sea urchin egg

By TRYGGVE GUSTAFSON and MAJ-BRITT HJELTE

The sea urchin egg is, during its earlier development, morphogenetically a very labile system. Thus the addition of lithiumions to the sea water in the earlier stages changes the morphogenesis profoundly, the entire egg sometimes developing into an endoderm. (HERBST 1892, see LINDAHL 1942.) If, on the other hand, the eggs are treated with thiocyanate ions in calcium-free sea water before fertilization the effect may be the reverse, the whole egg developing into an ectoderm vesicle (cf. LINDAHL 1942).

One may suppose that these ions interfere with the processes of synthesis upon which histogenesis is based. To get a further insight into the mode of action of these ions we carried out a study of the content of various amino acids in the development of both normal and Li-treated eggs.

Sea urchin eggs, *Paracentrotus lividus*, were collected at Station Biologique de Roscoff[1], Bretagne, France. Ninety-nine to a hundred per cent of the eggs could be fertilized. The eggs were cultivated in dilute suspensions in glass trays following the method of LINDAHL (1936). The temperature was always kept constant at 22° C and the stages taken for analysis were always chosen with reference to the respiration curve given by LINDAHL (1939).

It is known, see HÖRSTADIUS (1936), that a long Li treatment at a low concentration has the same effect as a shorter treatment with a higher concentration. For practical reasons longer treatment with a comparatively weak Li concentration was chosen, using 95 parts sea water of salinity 3.5 per cent and 5 parts isotonic Li solution. The treatment was always started immediately after fertilization and was interrupted after 4, 7, 12, 18 or 20 hours. If the larvae were cultivated for a longer time they were always washed repeatedly after 20 hours and transferred into fresh sea water. A 12 hour treatment under these conditions gave rise to strongly vegetalized larvae with only a small ectoderm vesicle. The eggs were centrifuged down at various stages and were frozen on a galvanized plate, previously chilled to —20° C. The material was then dried in the frozen state and stored in a vacuum desiccator over anhydrous silica gel.

For the analysis equal parts of three different preparations at the same stage were mixed and e.g. 250 mg were hydrolyzed in a closed pyrex glass ampoule with 3.5 ml 10 per cent HCl for 10 hours at 120°. This treatment seems to be sufficient for most purposes. For the analysed of tryptophane the hydrolyses were performed with NaOH which had been previously autoclaved with cysteine (Following KUIKEN et al. 1947) to protect the tryptophane from oxidation during the hydrolysis.

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[1] We wish to express our gratitude to the Director of the station, Professor G. TEISSIER, and to the Sous-directeur, Professor P. DRACH, for their never failing generosity.

The first analyses were performed by adsorption methods with activated charcoal and ion exchange resins (TISELIUS et al. 1947), and good preliminary results could be obtained by this method. Colorimetric methods were also tried, sometimes with reliable results. Neither of these methods, however, seemed to be very suitable for the analysis of numerous samples on the small scale which it was necessary to use. Therefore microbiological methods were tried with better results. When the results could be checked with other methods there was always excellent agreement. As these microbiological methods require only minute amounts of material they seem to be very well adapted to the present purpose.

The methods used were basically that of STOKES et al. (1945) and that of HENDERSON et al. (1948). Estimation of the growth was always made by means of titration, generally electrometrically. The analyses were generally made at 3 or 4 different concentrations, each concentration in triplicate. Several separate determinations were made for most of the acids and generally two different hydrolyzates were examined. The different results for the same amino acid generally showed excellent agreement. Sometimes however the magnitude was somewhat different in different series but the form of the curve was the same. The curves recorded are generally based on the mean of several series of determination. The amino acid nitrogen is expressed as per cent of the total (Kjeldahl) nitrogen of the egg. As the nitrogen content of the egg seems to be constant at least for the first 20 hours the changes in the different amino acids, up to this time, must be the result of real changes and not dependent upon or distorted by diminutions of the total nitrogen of the egg. This question will however be further examined.

As a complement to the microbiological methods the Van Slyke manometric method was used for determinations of amino nitrogen. Paper chromatographic methods were also used for the study of the free amino acids. Two-dimensional analyses were performed on desalted trichloroacetic acid extracts with collidine, and phenol on Munktell OB filter-paper.

Results

The results may be best described by the accompanying table. As will be seen from this the amino acid composition of the egg is remarkably constant before gastrulation. A more careful analysis of the figures shows, however, that a definate drop in the total amount of amino-nitrogen occurs during the first two hours after fertilization. The major part of the drop is due to a loss of glycine but there is also a clear drop in some of the other amino acids.

During gastrulation a more or less distinct drop in the content of various amino acids appears while glycine shows an increment. For certain amino acids no clear changes could be demonstrated.

Lithium in certain cases, e.g. valine, aspartic acid, leucine and tyrosine, does not change the picture of the normal development. In other cases more or less pronounced changes occur. Thus e.g. the contents of glutamic acid, alanine and arginine are already lower in the Li stages than in the normal stages after 4 or 7 hours. Another group of amino acids including cystine, methionine, glycine and serine seems to change less during the later stages than in normal development. In still other cases the situation needs further examination.

The low-molecular-weight nitrogen fraction extractable after protein precipitation with tungstic acid shows the remarkably high figure of 21 per cent of the total

Table 1

Amino acid-N in per cent of total-N (Kieldahl-estimations).

Amino acids	Minutes (m) respectively hours (h) after fertilization																			
	Normal development														Lithium development					
	0 m	10 m	30 m	1 h	2 h	4 h	6 h	7 h	8 h	9 h	12 h	18 h	30 h	48 h	4 h	7 h	12 h	18 h	30 h	48 h
Glycine	23.1	21.8	21.8	22.0	21.1	21.3	21.6	21.6	21.9	21.8	21.5	22.4	23.8	25.2	21.4	22.0	21.9	21.3	22.1	21.1
Alanine	3.53	3.57	3.57	3.66	3.57	3.67	3.43	3.57	3.58	3.40	3.43	3.32	3.62	4.11	3.24	3.42	3.40	3.22	3.38	3.66
Valine	5.18	5.21	5.08	5.12	5.12	5.07	5.08	5.08	5.17	5.06	5.01	4.89	4.76	4.67	5.15	5.09	5.16	4.78	4.73	4.55
Leucine	4.38	4.34	4.34	4.30	4.29	4.30	4.33	4.28	4.33	4.33	4.34	4.16	4.02	3.93	4.36	4.30	4.30	4.19	4.05	3.93
Isoleucine	3.82	3.77	3.82	3.75	3.72	3.78	3.71	3.72	3.82	3.76	3.70	3.53	3.57	3.60	3.79	3.82	3.65	3.61	3.32	3.22
Serine	5.14	4.98	4.87	4.86	4.66	4.94	4.78	4.77	4.46	4.56	4.30	4.18	4.16	4.15	4.82	4:68	4.42	4.30	4.22	4.15
Threonine	2.80	2.97	2.93	2.85	2.84	2.67	2.86	2.69	2.73	2.82	2.83	2.79	2.64	2.76	2.84	2.86	2.84	2.87	2.88	2.58
Cystine	0.77	0.75	0.74	0.71	0.73	0.76	0.75	0.68	0.69	0.68	0.65	0.58	0.64	0.65	0.72	0.72	0.67	0.65	0.62	0.58
Methionine	1.40	1.48	1.49	1.38	1.48	1.51	1.54	1.45	1.43	1.40	1.37	1.28	1.29	1.26	1.48	1.41	1.35	1.35	1.34	1.22
Phenyl-Alanine	2.02	2.08	1.97	2.00	2.00	2.00	2.06	1.93	2.00	2.07	1.99	1.94	1.83	1.92	2.04	1.93	1.93	1.92	1.93	1.78
Tyrosine	1.26	1.21	1.20	1.27	1.25	1.22	1.20	1.25	1.28	1.28	1.20	1.19	1.19	1.14	1.24	1.25	1.22	1.19	1.20	1.18
Tryptophane	0.67	0.66	0.62	0.66	0.63	0.61	0.64	0.66	0.65	0.64	0.63	0.63	0.63	0.55	0.64	0.62	0.62	0.62	0.60	0.57
Proline	2.25	2.49	2.32	2.24	2.42	2.26	2.37	2.37	2.37	2.37	2.30	2.08	2.08	2.32	2.32	2.32	2.39	2.24	2.43	2.25
Aspartic acid	5.68	5.60	5.68	5.57	5.49	5.64	5.60	5.45	5.48	5.51	5.21	5.14	4.94	4.60	5.59	5.55	5.30	5.19	4.80	4.71
Glutamic acid	5.54	5.62	5.53	5.45	5.52	5.62	5.57	5.58	5.33	5.33	5.07	5.06	4.99	5.03	5.39	5.33	5.19	5.03	5.04	5.11
Arginine	10.6	10.3	10.2	10.4	10.2	10.4	10.0	10.2	10.4	10.4	10.4	10.3	10.6	11.0	9.8	9.9	9.8	9.6	9.7	10.1
Lysine	6.92	6.75	6.90	6.94	6.72	6.51	6.49	6.45	6.68	6.69	6.45	6.36	6.18	6.03	6.68	6.73	6.45	6.17	5.84	5.51
Histidine	3.00	3.01	2.95	2.89	3.06	2.87	3.03	2.89	2.84	2.82	2.91	2.79	2.89	2.84	2.89	2.95	2.88	2.90	2.80	2.68
Total	88.1	86.6	86.0	86.1	84.8	85.1	85.0	84.6	85.1	84.9	83.3	82.6	83.8	85.8	84.4	84.9	83.5	81.1	81.0	78.9

nitrogen of the egg. According to paper chromatographic analysis most of this nitrogen occurs as glycine during the whole development, see ÖRSTRÖM (1941), but the presence of most of the other common amino acids can also be demonstrated.

The free amino nitrogen shows some reduction during gastrulation in normal stages, from 21 % to 17.6 %. This reduction can not be demonstrated in the Li development. The total (Kjeldahl) nitrogen of the extracts is however constant in both cases. This may indicate that free amino nitrogen disappears, perhaps by peptide formation, during the normal but not during the Li development.

It has been possible to demonstrate the presence of peptides in the extract by paper chromatography but their appearance at gastrulation needs further evidence.

Free glutamine has been demonstrated in the extracts at earlier stages. At normal gastrulation and at comparable Li stages the glutamine disappears almost entirely.

Discussion

It may be difficult to draw general conclusions from the data presented above. The period of normal determination seems however to show few changes in amino acid content. These appear just when differentiation begins. This is consistent with the studies of GUSTAFSON and HASSELBERG (1950) on alkaline phosphatase which shows very weak activity up to gastrulation, when a rapid rise occurs. It is also consistent with the studies in progress on the ATP-ase effect of the eggs, which is low up to gastrulation and then increases. The studies of PERLMANN and GUSTAFSON (1948) also show no appearance of new antigens in the egg until after the beginning of gastrulation.

The Li studies may indicate that the vegetal direction of determination is characterized by a breakdown of certain amino acids (e.g. glutamic acid, alanine and arginine). Under the influence of lithium the changes during differentiation in many cases seem to be less pronounced than during normal development. This is not only the case with the total amount of certain individual amino acids but also with the changes of the free amino nitrogen in protein free extracts. Studies in progress on ATP-ase activity are consistent with this picture as they indicate a slower increase at differentiation in the ATP-ase activity in the Li stages than in normal development. It may be that the Li development is characterized by a general checking of synthesis processes while the animal development is based on processes of intensive synthesis.

This work has been carried out with the aid of the Swedish Research Council, Liljewalch Jr.'s resestipendiefond, and Gustaf and Anna Retzius' fond för vetenskaplig forskning.

We wish to express our deep gratitude to Professor John Runnström for his never failing interest in our work, and to Miss Ingrid Hasselberg for her assistance with the collection of material and certain determinations. We also thank Mr Johannes Norman for technical assistance.

From the Wenner-Gren Institute for exp. Biology, University Stockholm.

REFERENCES. **Gustafson, T.** and **Hasselberg, I.** 1950. In press. — **Henderson—Snell.** 1948. J. Biol. Chem. *172*. — **Herbst, C.** 1892. Ztschr. Wiss. Zool., *55*. — **Hörstadius, S.** 1936. Arch. Entw.-mechan., 135. — **Kuiken, K. A., Lyman, C. M.** and **Hale, F.** 1947. J. Biol. Chem., *171*. — **Lindahl, P.-E.** 1936. Acta Zool., Stockholm, *17*. — **Lindahl, P.-E.** 1939. Ztschr. vergleichende Physiol., *27*. — **Lindahl, P.-E.** 1942. Quart. Rev. Biol., *17*. — **Örström, Å.**, 1941. Ark. Kemi, *15 A*. — **Perlmann, P.** and **Gustafson, T.** 1948. Experientia, *4*. — **Stokes, J. L.** and **Gunness, M., Dvyer, J. M.** and **Caswell, M. C.** 1945. J. Biol. Chem., *160*. — **Tiselius, A., Drake, B.,** and **Hagdahl, L.** 1947. Experientia, *3*.

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ARKIV FÖR KEMI Band 2 nr 23

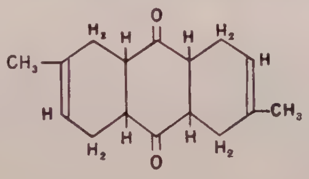
Vorgelegt am 12. April 1950

Elektronen-Transport und Stabilisierung bei der Reaktion zwischen Dienen und Philodienen. I

Von Hans v. Euler und Hans Hasselquist

Mit 1 Figur im Text

Eine Reaktion, die sich im Sinne der chemischen Elektronentheorie als Elektronen-Übertragung vom Dien auf die dienophile Substanz darstellt, ist von Euler und Josephson[1] 1920 aufgefunden und das Produkt ist (ohne Entscheidung über die Methylstellung im Produkt) folgendermaassen formuliert worden:



Die Einsicht in die zwischen Dien und Philodien eintretende Resonanz und in den bei dieser Gruppe von Vorgängen zunächst erfolgenden Elektronentransport ist offenbar von reaktionskinetischen Gesichtspunkten aus von grosser allgemeiner Bedeutung, und wir haben demgemäss das Studium des im hiesigen Arbeitskreis zuerst beschriebenen, später besonders von Diels und Alder[3] eingehend und erfolgreich untersuchten Reaktionstypus an anderen Paaren von Dienen und Philodienen wieder aufgenommen.

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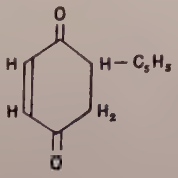
[1] Euler u. K. Josephson, Chem. Ber. 53, 822 (1920). — Euler u. Hasselquist, Kem. Arb. 13, Suppl. I (1950).

[2] Ein Additionsprodukt aus Cyklopentadien und Chinon hat 1906 W. Albrecht (Lieb. Ann. 348, 31; 1906) folgendermaassen formuliert:



Diese Formel ist von H. Staudinger (1912) und von O. Diels, H. Blom u. W. Koll (Lieb. Ann. 443, 242; 1925) kritisiert bezw. verbessert worden.

[3] O. Diels u. K. Alder, Lieb. Ann. 460, 98 (1928). — Chem. Ber. 62, 2348 (1929).

Die Stabilität bezw. Umlagerung der durch den Elektronen-Transport induzierten Produkte — wir verweisen hinsichtlich des Reaktionsmechanismus besonders auch die Arbeiten von BERGMANN und ESCHINAZI[1] von HUDSON und SIR R. ROBINSON[2] sowie von H. HENECKA[3] — verläuft an verschiedenen Dienen mit verschiedener Geschwindigkeit, und dieser Reaktionsphase haben wir besondere Aufmerksamkeit gewidmet.

Die in der vorliegenden Mitteilung beschriebenen Produkte sind mit 1,3-Dimethyl-Butadien[4] und mit p-Chinon als Philodien dargestellt worden.

Ausgangs-Produkt HH 105. Siehe Übersicht S. 369

Eine Mischung von 1 Mol. p-Chinon und 2 Mol. Dimethyl-butadien wurde erhitzt und dann vom Wasserbad abgenommen. Die Reaktionswärme der in Gang gesetzten Reaktion reichte dann hin, um die Mischung während mehrerer Minuten im Sieden zu erhalten. Das unverbrauchte Dimethyl-butadien wurde abdestilliert, der Rückstand mit Petroleumäther verdünnt; nach dem Abkühlen fielen grosse, gelbgrüne Prismen aus, die, mit Petroleumäther umkrystallisiert, bei 48°–50° schmolzen.

Analyse HH 105:	Für $C_{12}H_{14}O_2$				
		ber.	C = 75,76 %	H = 7,42 %	Mol. Gew. 190
		gef.	74,91 %	7,48 %	195

Ein aus p-Chinon und 1,3-Dimethyl-butadien erhaltenes Produkt ist in DRP 521 633 der I. G. Farbenindustrie A. G. kurz erwähnt. Obwohl der Schmpt., 60°, von dem unseres Produktes HH 105 erheblich abweicht, dürfte es sich um die gleiche Substanz handeln.

5,8-Dihydro-1,4-dioxy-5,7-dimethyl-naphtalin. $C_{12}H_{14}O_2$ HH 106
Umlagerung des Ausgangsproduktes HH 105

Versetzt man 2 g der Substanz HH 105, gelöst in 1 ml Eisessig, mit 1 Tropfen HBr-Eisessig-Lösung, so wird durch die Reaktionswärme der Siedepunkt der Lösung erreicht. Nach Abkühlung und Zusatz der 4-fachen Menge Wasser scheidet sich ein Öl ab, aus welchem farblose Nadeln von Schmpt. 104,5° gewonnen werden. In Eisessig gelöst zeichnet sich HH 106 durch dunkelviolette Fluorescenz aus.[5]

Analyse HH 106:	Für $C_{12}H_{14}O_2$			
		ber.	C = 75,76 %	H = 7,42 %
		gef.	75,80 %	7,35 %

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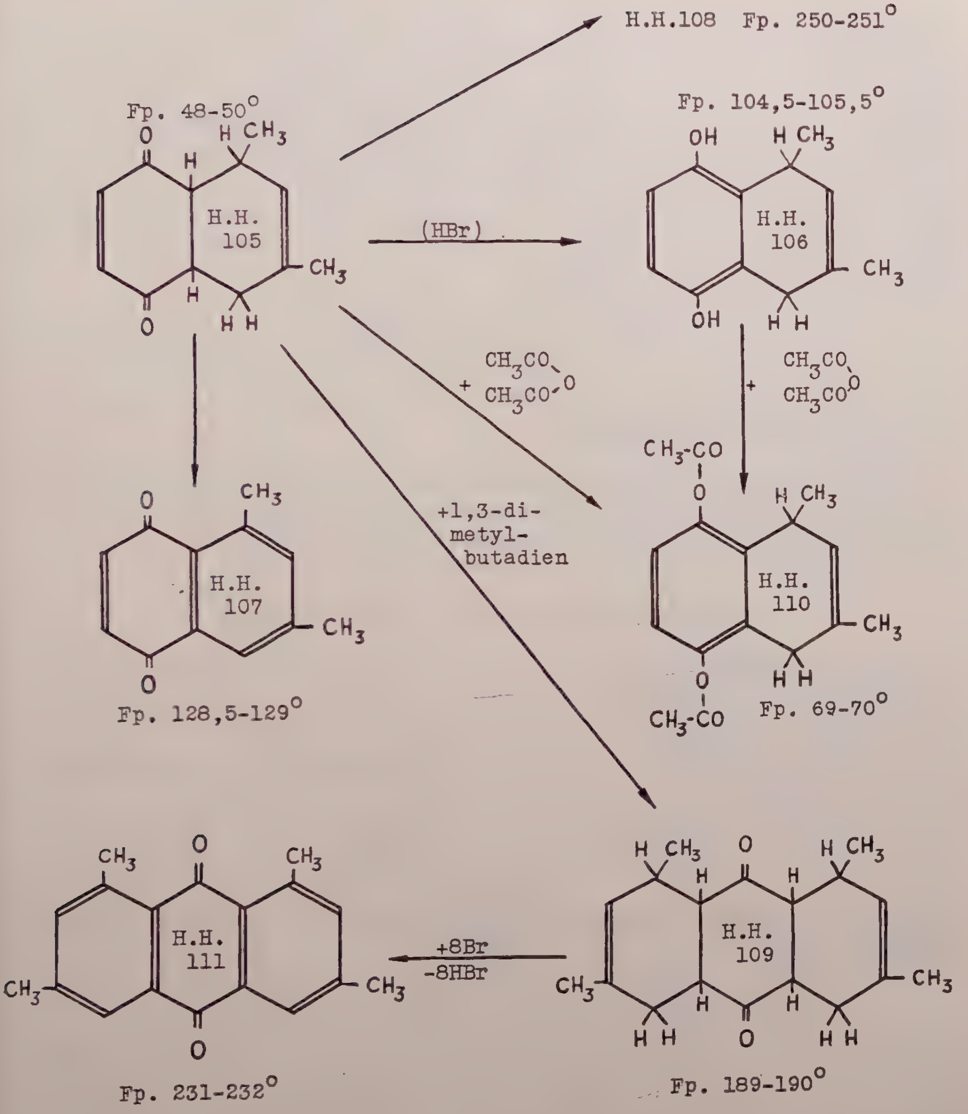
[1] FELIX BERGMANN u. H. E. ESCHINAZI, J. Am. Chem. Soc. 65, 1405 (1943).

[2] B. J. F. HUDSON u. Sir R. ROBINSON, J. Chem. Soc. 1941, 715.

[3] H. HENECKA, Z. f. Naturforsch. 4 b 15 (1949).

[4] Wir sind Herrn Direktor Dr. Dr. h. c. WALTER REPPE, Bad. Anilin-u. Soda-Fabrik, Ludwigshafen, für die Überlassung einer Reihe von Dienen zu grossem Dank verpflichtet.

[5] Dieser Übergang 105→106 entspricht der von DIELS und ALDER (Ber. 62, 2337; 1929) beschriebenen Umlagerung von Mono-butadien-chinon in 1,4-Dihydro-5,8-dioxy-naphtalin, wobei die beiden γ-Wasserstoffatome entfernt werden.


H.H.108 Fp. 250-251°
Fp. 48-50°
H.H. 105
(HBr)
Fp. 104,5-105,5°
H.H. 106
CH3CO
CH3CO O
+1,3-dimetylbutadien
H.H. 107
Fp. 128,5-129°
CH3-CO
H.H. 110
Fp. 69-70°
H.H. 111
+8Br
-8HBr
H.H. 109
Fp. 231-232°
Fp. 189-190°

Acetylierung der Subst. HH 106. Diese Acetylierung lässt sich mittels Essigsäureanhydrid + H_2SO_4 in der Wärme leicht durchführen. Das Diacetylprodukt, HH 110, bildet farblose Prismen, die bei 69°–70° schmelzen.

Analyse HH 110:	Für $C_{16}H_{18}O_4$	ber.	CH_3CO— 31,38 %	Mol. Gew.	274
		gef.	27,80 %		270

Oxydation des Ausgangsproduktes. Substanz 107

2 g des in 10 ml Eisessig gelösten Ausgangsmateriales HH 105 (vgl. S. 369) wurden unter Zusatz von 1,6 g CrO_3 bis zum Eintritt der Reaktion auf dem Wasserbad vorsichtig erwärmt. Nach Ablauf der Reaktion bei Zimmertemperatur wird mit 100 ml Wasser verdünnt und die Lösung mit Petroleumäther ausgeschüttelt. Aus den eingeengten und abgekühlten Extrakten entsteht das Oxydationsprodukt (0,7 g) als gelbe Nadeln. Durch Sublimieren derselben bei 120° gewinnt man die Substanz 107 in Form goldglänzender Nadeln vom Schpt. 128,5–129°. Dieselben lösen sich leicht in den meisten organischen Lösungsmitteln, schwer dagegen in Wasser.

Analyse HH 107:	Für $C_{12}H_{10}O_2$	ber.	C = 77,40 %	H = 5,41 %
		gef.	77,33 %	5,56 %

Die so erhaltene Subst. 107 ist als dem Ausgangsprodukt entsprechende Dimethyl-naphtochinon anzusehen.

Wurde die mit Wasser verdünnte Reaktionslösung mit Äther oder Benzol ausgeschüttelt, so konnte ausser dem Naphtochinon 107 auch eine chinhydron-artige Substanz in Form schwarzvioletter, in Petroleumäther schwerlöslicher Nadeln isoliert werden.

Bis-(dimethyl-butadien)-chinon; HH 109

Durch 2-stündiges Erhitzen von 2 g des Ausgangsproduktes HH 105 mit 1,2 g frischdestillierten 1,3-Dimethyl-butadiens im Bombenrohr bei 65° erhält man das Bis-(dimethyl-butadien)-chinon. Das entsprechende Bis-isopren-chinon entsteht direkt durch Behandlung von p-Chinon mit einem Überschuss von Isopren. Sbst. HH 109 fluoresciert in Eisessiglösung hellblau. Schpt 189–190°

Analyse HH 109:	Für $C_{18}H_{24}O_2$	ber.	C = 79,37 %	H = 8,88 %
		gef.	78,69 %	8,71 %

Die Stellung der Methylgruppen in den Substanzen 109 und 111 ist noch unbestimmt.

Oxydation der Sbst. HH 109 mit Brom

0,9 g Sbst. 109, in 10 ml suspendiert, wurde allmählich mit einer Lösung von 1 g Brom in 5 ml Benzol versetzt, bis die Bromfärbung bei Zimmertemperatur nicht mehr verschwand. Es schieden sich dann hellgelbe Krystalle ab, welche abfiltriert und mit Benzol gewaschen wurden, durch 2-maliges Umkrystallisieren des Rohproduktes (200 mg) aus Eisessig wird die halogenfreie, reine Substanz HH 111 in Form langer, hellgelber Nadeln vom Schmpt. 231–232° gewonnen. Durch Sublimieren bei ca 200° entstehen kleine gelbe Prismen. Die Sbst. HH 111 ist schwerlöslich in allen gewöhnlichen organischen Lösungsmitteln, unlöslich in Wasser.

Analyse HH 111:	Für $C_{18}H_{16}O_2$	ber.	C = 81,79 %	H = 6,10 %
		gef.	80,41 %	6,12 %

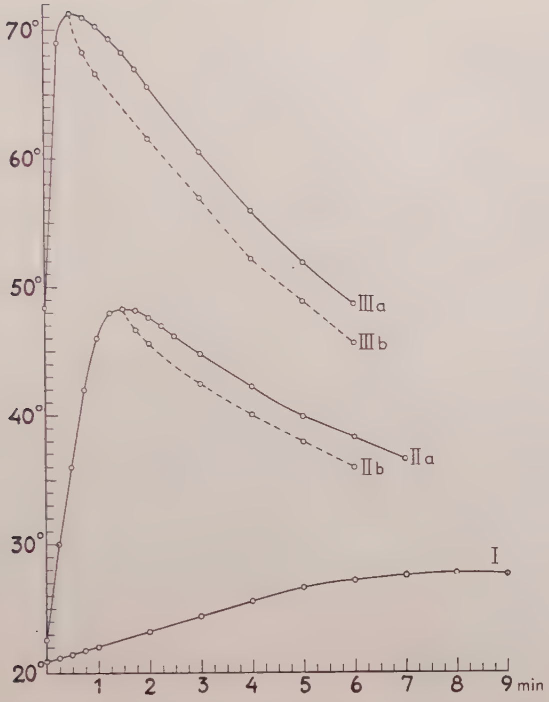


Fig. 1.

Wärme-Entwicklung bei der Umlagerung von HH 105 in HH 106

Seite 368 wurde bereits erwähnt, dass der Übergang der Sbst. HH 105 in HH 106 unter starker Temperatursteigerung der Reaktionslösung, also unter starker Wärme-Entwicklung verläuft. Wir haben diese Temperatursteigerung in orientierenden Messungen folgendermaassen ermittelt:

Ein Glasrohr 13 mm Durchmesser, in welchem sich die Reaktionslösung befand, war von einem weiteren Glasrohr umgeben, sodass ein 3 mm dicker Luftmantel die Wärmeabgabe des inneren Reaktionsgefässes verzögerte.

Bei den Versuchen I, II a und III a waren die Lösungen der Sbst. HH 105 mit einer Eisessiglösung von HBr versetzt, welche 0,13 mg freies HBr enthielt.

Versuch I. 300 mg HH 105 auf 2,0 ml Eisessig-HBr-Lösung.
Versuch II a. 600 mg HH 105 » » » » »
Versuch III a. 600 mg HH 105 » » » Eisessig-Lösung. Diese Lösung wurde auf 48,3° erwärmt, dann mit HBr-Eisessig versetzt.

In Parallelversuchen II b und III b, mit den HH 105-Konzentrationen II und III, jedoch ohne HBr, wurde der zeitliche Temperatur-Abfall gemessen.

Der Verlauf der Temperatursteigerung ist in der Figur 1 angegeben, in welcher die Bezeichnung der Kurven den oben angegebenen Versuchen entspricht.

In erster Linie ist darauf hinzuweisen, wie stark die Temperatursteigerung *mit der Konzentration der Sbst. HH 105* zunimmt: In Versuch I trat das Temperaturmaximum (rund + 5°) erst nach 9 Minuten ein, während im Versuch II das Temperaturmaximum (+ 25°) schon nach 1,5 Minuten erreicht wurde. Bei beiden Versuchen war das Mengenverhältnis von HBr zu HH 105 das gleiche, nur das Verhältnis ihrer Mengen zur Menge Lösungsmittel war geändert. Der grosse Unterschied zwischen den Kurven I und II lässt sich wohl z. T. auf eine Verschiebung des Gleichgewichtes Sbst. $105 \rightleftarrows HBr$ zurückführen; auch ist in Betracht zu ziehen, dass die Umwandlung, deren Wärmeeffekt wir messen, nicht monomolekular, sondern polymolekular verläuft.

Dass bei der Anfangstemperatur 48,3° der Anstieg zum Temperaturmaximum sehr viel rascher (in ca 30 Sekunden) erreicht wird als bei der Anfangstemperatur 22,4°, entspricht einem Temperaturkoeffizienten der Reaktionsgeschwindigkeit, der noch höher erwartet werden konnte.

Sbst. HH 105 ist in Abwesenheit von starken Säuren stabil, und zwar auch in der Wärme und im ultravioletten Licht. Wenn sich HBr oder HCl an HH 105 addiert, so werden die angulären H-Atome gelockert.

Es bleibt dann noch die Tatsache zu betonen, dass nicht nur die eben erwähnte Umwandlung von 105 in die stabilere Sbst. 106, sondern auch die zur Sbst. 105 führende Diensynthese mit einer starken Wärme-Entwicklung verbunden ist.

Das wesentlichste Problem, das sich in diesem Zusammenhang ergibt, betrifft die Beziehung zwischen der die katalysierte Umlagerung vermittelnden Elektronen- und Atom-Verschiebung und dem dadurch veranlassten Wärme-Effekt. Das Studium der entsprechenden Reaktion an anderen Dienen und Philodienen wird vielleicht zu einer diesbezüglichen Aufklärung beitragen.

Stockholm, Universität, Inst. f. organ.-chem. Forschung und Vitamin-Institut.

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Zur Kenntnis der Reduktone. VI

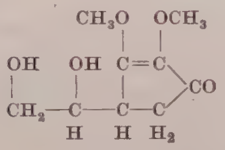
Von Hans v. Euler und Hans Hasselquist

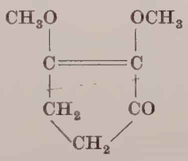
Die Methylierung eines Endiol-Hydroxyls lässt sich bekanntlich mittels Diazomethan leicht durchführen. Ein Triose-redukton-monomethyläther wurde bereits von Martius[1] in Form farbloser Blättchen von Schmpt. 67° erhalten. Welche der beiden Endiolhydroxyle darin veräthert ist bleibt noch endgültig aufzuklären.

In dieses Redukton ist bisher ein zweites Methyl noch nicht eingeführt worden.

Dagegen sind Di-methyläther *anderer* Endiole bekannt; wir erwähnen die in 2, 3-Stellung methylierten[1] Ascorbinsäure-derivate (I), welche in den Stellungen 2, 3, ferner 2, 3, 5 und 2, 3, 5, 6 methyliert sind.[2]

I





II

Genannt seien ausserdem noch der Di-methyläther[3] der Reduktinsäure[4] (II).

Bemerkenswert ist die leichte Spaltbarkeit der aliphatischen Monomethyläther durch verdünntes Alkali; man sieht, dass der Charakter der Endiolhydroxyle gegenüber sonstigen Alkohol-hydroxylen stark verändert ist.

Unter den Di-methyläthern *aromatischer* Endiole ist zunächst das Veratrol, der Di-methyläther des Brenzcatechins zu nennen, der durch Behandlung von Guajakol mit Dimethylsulfat gewonnen wird. Ferner kennt man eine erhebliche Anzahl von natürlich vorkommenden Di-methyläthern des Brenzcatechins sowie des Pyrogallols und ihrer Derivate (Elemicin, Asaron u. a.). Als Di-methyläther eines *heterocyklischen* Reduktons sei der von Arndt und Eistert[5] dargestellte Thiochromondiol-di-methyläther angeführt.

Die sterische Lage der beiden Endiol-hydroxyle gestattet, neben der Methylgruppe eine Acetylgruppe einzuführen. Durch Behandlung des Monoacetyl-reduktons mit Diazomethan entsteht das früher nicht beschriebene *Monomethyl-redukton-acetat*, ein farbloses Öl.

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[1] Euler u. Martius, Lieb. Ann. 505, 73 u. zw. 85 (1933).

[2] W. N. Haworth, E. L. Hirst u. F. Smith, Soc. 1934, 1556. — 1937, 839. — Siehe hierzu auch die Übersicht von F. Smith, Advances in Carbohydrate Chemistry 2, 79 (1946).

[3] H. J. H. Fenton u. W. A. R. Wilks, Soc. 101, 1970 (1912).

[4] T. Reichstein u. R. Oppenauer, Helv. 16, 988 u. zw. 997 (1933).

[5] F. Arndt u. B. Eistert, Ber. 62, 36 (1929).

Darstellung: 50 mg Acetyl-Verbindung wurde mit einer ätherischen Lösung von Diazomethan versetzt. Es trat sofort eine kräftige Gasentwicklung auf, die im Verlauf von 10 Min. beendet war. Nach Eindunsten der Reaktionslösung im Vacuum bei 20° blieb ein farbloses Öl zurück das sich in Alkohol, Äther, Aceton und Chloroform leicht löst. Es liefert mit $FeCl_3$ in alkoholischer Lösung keine Farbenreaktion.

Der Methylen-äther des Triose-reduktons dürfte bei einem von Martius[1] angestellten Versuch aus Redukton und Formaldehyd entstanden sein, worauf das Verschwinden des Reduktons schliessen lässt.

I. Ester des Triosereduktons

Acetylierung der beiden Endiolhydroxyle ist durchgeführt worden u. a. bei Ascorbinsäure-Derivaten[2] ferner mittels Essigsäureanhydrid und Acetylchlorid an Reduktinsäure.[3] Zur Charakterisierung der aliphatischen Endiolgruppe haben wir die Veresterung mit ein- und zweibasischen Säuren und das Verhalten der isolierten Ester des Triosereduktons studiert.

Triosereduktion-acetat

Nachdem ein mit Redukton angestellter Acetylierungsversuch nur zu harzigen oder öligen Produkten geführt hatte, gelang die Darstellung eines krystallisierten Acetates wenn von *reinstem* Na-Salz des Reduktons ausgegangen wurde. Das nach unserer Vorschrift in Arkiv 26 B Nr 8. S. 7, Anm. dargestellte Salz (1 g) wurde in 20 ml wasserfreien Benzols aufgeschlämmt und mit 0,64 ml Acetylchlorid versetzt durch vorsichtiges Erwärmen kam die Reaktion in Gang; durch die Reaktionswärme wurde dann das Benzol im Sieden erhalten. Nach Erkalten und Filtrieren wird die Lösung im Vacuum eingeengt, wobei die Acetylverbindung in Form grosser farbloser Prismen ausfallen. Nach Abfiltrieren und Waschen mit wenig Benzol und Trocknen schmolz das Rohprodukt (400 mg) bei 99–101° ohne Zersetzung.[4] Hält man die Schmelze 15 Min. bei 106°, so entsteht *nicht*, wie bei der Erhitzung der Monochlor-Verbindung (vgl. S. 375) eine feste Substanz.

Die Sbst. ist leicht löslich in Alkohol, Aceton, Eisessig und Benzol, schwerer in Wasser. Die alkoholische Lösung färbt sich auf Zusatz von $FeCl_3$ rotbraun.

Bei der Titration mit 0,01 n. Lösung des Tillmans-Indikators (TR) bei 20° wurden von 4,1 mg Sbst. 6,6 ml verbraucht. Für die Monoacetylverbindung $C_5H_6O_4$[1] berechnet sich $E = 65$, Gefunden $4{,}1/0{,}01\ 0{,}6 = 62$.

Analyse:	Berechnet für $C_5H_6O_4$	$C = 46{,}16$ %	$H = 4{,}65$ %
	Gefunden	46,60	4,92

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[1] Euler u. C. Martius, Lieb. Ann. 505, 87 (1933).

[2] Siehe F. Smith, Adv. in Carbohydrate Chem. l. c.

[3] T. Reichstein u. R. Oppenauer, Helv. 17, 390 (1934).

[4] Die Sbst. reagiert mit Diazomethan unter lebhafter Stickstoff-Entwicklung, was, ebenso wie die Färbung mit $FeCl_3$ die Gegenwart eines freien Hydroxyls anzeigt.

Triosereduktondchloracetat

Da es sich in einem Vorversuch gezeigt hatte, dass in der Wärme ein primär entstehendes Produkt schnell in eine höher schmelzende Substanz übergeht, haben wir die Veresterung in der Kälte ausgeführt.

Mischt man 1 g Redukton mit 4 ml Chloracetylchlorid so tritt bald unter Gasentwicklung eine Kondensation ein, und nach Abschluss der Reaktion fallen farblose Prismen aus. Dieselben wurden in der kleinsten möglichen Menge Aceton gelöst und 30 Min. mit aktiver Kohle behandelt. Nach weiteren 30 Min. wurde die Reaktionsmasse abfiltriert und im Vakuum bei 20° bis zur Trockene eingedunstet.

Der Rückstand wurde mit 20 ml Benzol *rasch* aufgekocht, wobei sich an den Gefässwänden ein Harz absetzte, von dem abgegossen wurde. Aus der Lösung schieden sich grosse farblose Prismen vom Schmpt 95–102° ab, welche bei weiterem Umkrystallisieren aus Benzol ihren Schmpt unverändert beibehielten.

Hält man die Masse etwa 1 Min. an der oberen Grenze des Schmelzintervalles, so erstarrt die Schmelze und schmilzt dann den unter starker Zersetzung im Temperaturgebiet 120–130°.

Die Substanz ist schwerlöslich in Benzol, Petroleumäther und Wasser, leicht löslich in Alkohol oder Aceton. Sie liefert mit $FeCl_3$ in Alkohol eine intensiv rote Färbung.

Analyse: Eine Chlorbestimmung ergab Cl = 21,76 %.

Für Redukton-monochloracetat, $C_5H_5O_4Cl$ ber. Cl = 21,55 %.

2,6 mg Sbst. verbrauchten in alkalischer Lösung 3,3 ml 0,01 n. TR-Lös.

Gef. $2{,}6/0{,}01 \times 3{,}3 \quad E = 79$
Ber. für $C_5H_5O_4Cl \quad E = 82$

Wir nehmen an, dass hier das durch Verseifung zurückgebildete Redukton reagiert hat.

Erhitzungsprodukt des Triosereduktondchloracetates

500 mg des Chloracetates wurden in einem Reagenzglas im Paraffinbad unter ständigem Rühren 10 Min. auf 100° erwärmt. Es fiel dabei eine mikrokrystallinische Substanz in Form kleiner farbloser Nadeln aus. Die Schmelze wurde mit abs. Alkohol versetzt, worin das Erhitzungsprodukt sich nicht löste. Da das Ausgangsmaterial in Alkohol leicht löslich ist, konnten die beiden Stoffe leicht getrennt werden.

Die Ausbeute an Substanz S betrug 80 mg; sie schmolz bei 200° ohne Zersetzung.

Substanz 92 ist schwerlöslich in Wasser, Alkohol, Eisessig, Benzol, Äther und Chloroform; äusserst leicht löslich in verdünntem Alkali; durch Neutralisation fällt sie wiederum in kleinen Nadeln von obigem Schmpt 200° aus.

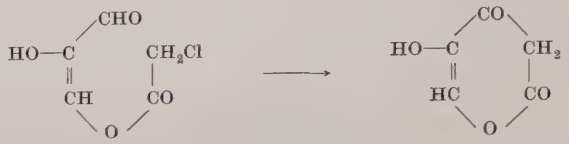
Suspendiert man Subst. 92 in Wasser und lässt die Suspension etwa 24 St. bei 20° stehen, so geht sie vollständig in Lösung, anscheinend unter Hydrolyse. Die Sbst. S 92 ist halogenfrei, reagiert mit Diazomethan verbraucht, aufgeschlämmt in Chloroform auch Brom.

Bei der Titration in alkalischer Lösung mit 0,01 n. TR-Lösung verbrauchten 4,6 mg Sbst. 7,65 ml.

Es wurde also gefunden: $E = 4{,}6 / 0{,}01 \times 7{,}65 = 60$

Berechnet für $C_5H_4O_4$ $E = 64$

Wir zogen zunächst in Betracht, dass sich in der Schmelze die Sbst. 89 (III) in folgender Weise in Sbst. 92 (5-Oxy-pyronon 2,4) verwandelt, die in Alkali und auch in Wasser verseift wird.


III → IV

Analyse:			
Berechnet für $C_5H_4O_4$		C = 46,88 %	H = 3,15 %
Gefunden		45,16	3,96
Berechnet für $C_6H_6O_5$		45,5	3,80

Für die Formel IV stimmen die gefundenen Werte also nicht. Für die Formel $C_6H_6O_5$ können wir einstweilen noch keine Struktur angeben.

Um die Struktur der Sbst. 89 sicherzustellen wurde dieselbe, wie früher Redukton[1] mit o-Phenylendiamin gekoppelt.

Darstellung: 500 mg Sbst. 89 in 2 ml abs. Alkohol gelöst wurde mit 450 mg festem o-Phenylendiamin versetzt. Durch die unter Wärmeentwicklung verlaufende Reaktion färbte sich die Lösung rot und liess kleine schmutzig-gelbe Prismen fallen. Die nach Abkühlung der Lösung ausgeschiedenen mikroskopischen Krystalle wurden mit Alkohol gewaschen und schmolzen nach dem Trocknen bei 105–106°. Bei Reinigungs-Versuchen durch Umkrystallisation wurden nur ölige oder amorfe Produkte erhalten.

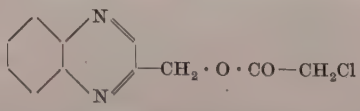
Subst. 91 enthält Cl, ist in Chloroform, Äthylacetat und Benzol schwer löslich, in Eisessig und noch mehr in Mineralsäuren leicht löslich.

Ein salzsaures Salz der Sbst. 91 wurde erhalten durch Zusatz von wenig konc. HCl in etwas Alkohol; dabei ging alles mit gelbbrauner Farbe in Lösung. Auf Zusatz von Äthylacetat schieden sich innerhalb einer Stunde dunkel-gelbgrüne viereckige Prismen ab, die abfiltriert und mit Äthylacetat gewaschen wurden. Schmpt. 220° unter starker Zersetzung.

Zwecks Cl-Bestimmung wurde Sbst. 91 einer Alkali-Schmelze unterworfen; die geschmolzene Masse wurde mit Wasser ausgekocht, die Lauge wurde mit Salpetersäure angesäuert und Cl mit $AgNO_3$ gefällt. Für $C_{11}H_9N_2O_2Cl$

Gef. 14,23 % Cl ber. 14,98 % Cl

Es hatte sich also, wie erwartet, das Chinoxalinderivat $C_{11}H_9N_2O_2Cl$ gebildet.



Aus der Entstehung eines Chinoxalinderivates kann auf die Struktur des Ausgangsmateriales geschlossen werden.

Methylierung der Sbst. 89 mit Diazomethan

Sbst. 89 soll nach Formel III ein freies aliphatisches Hydroxyl enthalten. Den Beweis dass dies der Fall ist, lieferte die Behandlung mit Diazomethan.

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[1] EULER, H. HASSELQUIST und U. LÖÖV, Dieses Arkiv 26 A Nr 17 (1948).

Die nach der üblichen Methode ausgeführte Methylierung lieferte zunächst ein hellgelbes, in Äther, Alkohol und Benzol leicht lösliches Öl, das sich bald zu einem gelben Harz polymerisierte. Beim Stehen des Öles in alkalischer Lösung wird *langsam* Redukton abgespalten.

Die alkoholische Lösung des Öles (Sbst. 90) färbt sich durch Zusatz von $FeCl_3$ nicht mehr; eine freie Hydroxylgruppe ist also nicht mehr vorhanden.

Das im Vacuum scharf getrocknete Öl wurde zwecks Cl-Bestimmung einer Alkali-Schmelze unterworfen.

Für $C_6H_7O_4Cl$

Gef.	20,77 % Cl
ber.	19,86 % Cl

Kondensation von Redukton mit Bernsteinsäure

Zu der Lösung von 440 mg Redukton in 1,6 ml Pyridin werden 1,5 g Bernsteinsäure-dichlorid (gelöst in 10 ml Benzol) unter Eiskühlung zugesetzt. Es fiel sofort eine pulverförmige Substanz aus, welche, mit Benzol gewaschen und getrocknet in 100 ml abs. Alkohol suspendiert wurde. Nach 2 Stunden ging das gebildete Pyridinhydrochlorid in Lösung. Nach Wiederholung dieses Reinigungsverfahrens bildet die Substanz ein Cl-freies, farbloses Pulver. Schmpt $> 300°$. Leichtlöslich in Wasser, aber schwerlöslich in allen gewöhnlichen organischen Lösungsmitteln.

Beim Schmelzen der Sbst. über freier Flamme entsteht unter Zersetzung ein leicht pulverisierbares Harz. Lässt man die wässrige Lösung der Sbst. frei verdunsten, so bleibt ein durchsichtiger Harzfilm zurück, der die polymere Beschaffenheit der Substanz anzeigt.

2,4 mg Sbst. in 10 ml Wasser gelöst, wurden mit 1 ml 2-n. NaOH im Stickstoffstrom versetzt und dann mit 0,01 n. TR-Lös. bei 60° titriert. Aus dem Verbrauch von 0,9 ml TR ergibt sich $E = 2{,}4 / 0{,}01 \cdot 0{,}9 = 267$. Da E einer Endiol-gruppe entspricht ergibt sich als Mindest-Mol. Gew. 534. Man kann daraus schliessen, dass das Redukton in der polymeren Substanz so gebunden ist, dass nur ein Teil davon durch Alkali freigemacht werden kann.

10,0 mg Sbst. in 5 ml Wasser gelöst, wurde mit 2 ml 0,0407-n Natronlauge bei Zimmertemp. stehen gelassen, worauf die Lösung mit 0,1 n HCl zurücktitriert wurde. Aus dem Verbrauch von NaOH berechnet sich

$$E = 195.$$

Dieses Ergebnis kann so gedeutet werden, dass bei der Einwirkung von Alkali Bruchstücke freigemacht werden können, welche auf 1 Mol Redukton 1 Mol Bernsteinsäure mit einer freien Carboxylgruppe enthalten.

Kondensation von Redukton mit Adipinsäure

Nicht nur die (aliphatischen) Endiol-äther werden durch verdünntes Alkali bemerkenswert leicht gespalten, sondern auch die Endiol-Ester. Dies zeigen die oben beschriebenen Reduktonester der Essigsäure, Chloressigsäure und Bernsteinsäure. Ähnlich verhält sich in dieser Hinsicht auch der Adipinsäure-ester des Reduktons.

Darstellung. 0,6 g Redukton in 2 ml Pyridin + 2 ml Benzol gelöst, wurde tropfenweise mit einer Lösung von Adipinsäure-dichlorid in 30 ml Benzol in der Kälte versetzt. Das sofort ausfallende Öl wurde nach 1 Stunde abdekantiert und mit Benzol gewaschen. Es wurde dann mit Wasser gewaschen und im Vacuum getrocknet. Zum Krystallisieren konnte es nicht gebracht werden.

Das Öl wurde nun mit einer konc. wässrigen Lösung von Semicarbazidchlorid + NaAc im Überschuss versetzt und blieb 48 Stunden bei Zimmertemperatur stehen. Die sich absetzenden Krystalle wurden abfiltriert, gewaschen und getrocknet und schmolzen dann bei 243° unter Zersetzung. Der gleiche Schmelzpunkt wurde erhalten nach Mischung mit Redukton-tri-semicarbazon. Aus dem Öl war durch die Einwirkung des Semicarbazids Redukton abgespalten worden.

Bei der Untersuchung von Äthern und Estern der Reduktone sind für uns hauptsächlich einige strukturchemische Gesichtspunkte und die Betrachtung der Endiolgruppe im Licht der chemischen Elektronentheorie massgebend gewesen. Wir werden auf diese Probleme sowie auf die Cis-Trans-Isomerie der Reduktone bald zurückkommen.

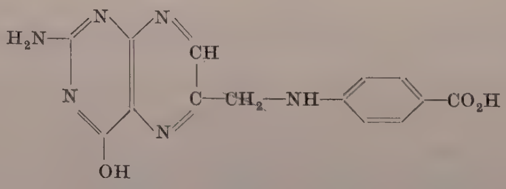
II. Kondensationsprodukte der Reduktone mit Aminosäuren

Ein aus Triose-redukton und einer aliphatischen Aminosäure, nämlich l-Leucin gewonnenes Produkt wurde vor kurzer Zeit von uns[1] beschrieben.

Die Umsetzung dieses Reduktons mit p-Aminobenzoesäure ist von besonderem biochemischen Interesse. Dass sowohl diese Stammsubstanz als Sulfonamid, Sulfapyridin und Sulfathiazol mit Redukton sich zu gefärbten, krystallisierenden Verbindungen kondensieren lassen, wurde von O'Meara, McNally und Nelson[2] angegeben, welche das Verhalten von Bakterien zu diesen Stoffen untersucht haben. Sie zogen in Betracht, dass die stark reduzierende, nicht Sh-haltige Substanz, welche in Kulturen von Bacterien während ihrer logarithmischen Entwicklungsphase auftritt, Redukton ist und dass p-Aminobenzoesäure im bacteriellen Stoffwechsel als Stabilisator fungiert. Die gleichen Forscher fanden auch, dass die p-Aminobenzoesäure-Redukton-Verbindung ein Wachstumsstoff für Streptococcen ist und dass Sulfonamide das bacterielle Wachstum hemmen, indem sie die p-Aminobenzoesäure dem Redukton entziehen.

Die erwähnte Annahme von O'Meara wurde von B. S. Forrest und J. Walker[2] zum Ausgangspunkt für weitere Studien über Redukton-Verbindungen gemacht.

Forrest und Walker erwähnen die Möglichkeit, dass im normalen bacteriellen Stoffwechsel p-Aminobenzoesäure und Redukton sich zunächst vereinigen und dann in die Pteroylsäure und in die anderen Faktoren dieser Gruppe eingebaut wird, während bei der Bacteriostasis durch Sulfonamid dieses sich mit dem Redukton verbindet und das Kondensationsprodukt in ein biologisch inaktives Analogon der Pteroylsäure eingeht.



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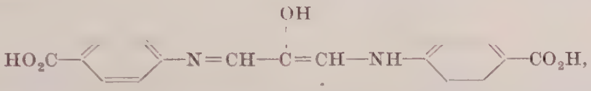
[1] Euler, H. Hasselquist u. Lööv, Arkiv för Kemi 26 A Nr 17, 8 (1948).

[2] H. S. Forrest u. J. Walker, Nature, 161, 721 (1948).

O'Meara und Mitarb.[1] teilten schon 1944 die Bildung eines krystallisierenden Produktes aus Redukton und p-Aminonenzoesäure mit, machten aber keine Angaben über die Zusammensetzung und die Eigenschaften dieser Substanz.

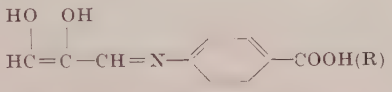
Forrest und Walker[2] erwähnen in ihren preliminären Versuchen als ersten Reaktionstyp die Vereinigung von Redukton und p-Aminobenzoesäure; sie beschreiben die Verbindung



und betonen, dass es für die Darstellung ihres Produktes wesentlich ist, von einer Mischung Redukton: p-Aminobenzoesäure im Verhältnis 1 : 2 auszugehen.

Den Beweis dafür, dass diese Substanz bei Anwendung des genannten Molekularverhältnisses gebildet wird sahen die Autoren in ihren analytischen Ergebnissen (l. c. S. 2005).

Schon vorher hatten Angier *et al.*[3] eine systematische Untersuchung ausgeführt über Derivate der aus 1 Mol Redukton und 1 Mol p-Aminobenzoesäure bestehenden Grundsubstanz



In den von dem amerikanischen Forscherkoncern beschriebenen Derivaten ist das Carboxyl-Hydroxyl ersetzt durch 6 verschiedene Gruppen R. Für die Grundsubstanz selbst werden folgende Daten angegeben:

Schmpt 255–258°. Ausbeute 90 %. Analyse:

Berechnet	57,96 % C	4,30 % H	6,76 % N
Gefunden	57,97	4,46	6,72

A. Bei unseren eigenen Versuchen über die Kombination von Redukton mit p-Aminobenzoesäure gingen wir zunächst, wie Forrest und Walker, vom Molekularverhältnis 1 Redukton, 2 p-Aminobenzoesäure aus, die in warmer 30 %-iger Essigsäure gelöst wurden. Fast unmittelbar fällt das gut krystallisierende Reaktionsprodukt in Form kleiner gelbrot gefärbter Nadeln aus. Nach mehrstündigem Verweilen im Kälteschrank wurde das Produkt abfiltriert, mit Essigsäure, Wasser, Alkohol und Äther gewaschen. Aus 0,44 g Redukton und 1,37 g p-Aminobenzoesäure erhielten wir 1,69 g Rohprodukt vom Schmpt. 248–254°. Bei etwa 210° färbt sich die Substanz dunkel. Das in allen üblichen Lösungsmitteln schwer lösliche Produkt wurde in folgender Weise gereinigt: Es wurde mit Pyridin auf dem Wasserbad erwärmt, wobei ein rotes Additionsprodukt entsteht, das abgeschieden und mit Äther gewaschen wurde. Beim Aufkochen mit Wasser tritt Hydrolyse ein; nach Auswaschen der abfiltrierten Substanz mit Wasser, Alkohol und Äther zeigt die nun reiner gelbe Substanz den Schmpt 264°; ein Mischschmelzpunkt mit der ursprünglichen Substanz gab aber keine Depression.

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[1] O'Meara, P. A. McNally u. H. G. Nelson, Nature, 154, 796 (1944). —

[2] H. S. Forrest u. J. Walker, Soc. 1949, 79 u. 2002.

[3] R. B. Angier et. al., J. Am. Chem. Soc. 70, 25 (1948).

B. Bei einem zweiten Versuch war die Reaktionsmischung 1 Mol Redukton auf 1 Mol p-Aminobenzoesäure; als Lösungsmittel wurde wiederum 30%ige Essigsäure verwendet. Die Ausbeute aus 0,22 g Redukton und 0,34 g p-Aminobenzoesäure betrug 0,497 und stimmte also durchaus mit der in Versuch A erhaltenen Ausbeute überein.

Ausbeuten:	Theoretisch	0,515 g
	nach A	0,44
	nach B	0,497

Es bestand nun noch die Möglichkeit, dass das abweichende Resultat von Forrest und Walker dadurch veranlasst war, dass diese Forscher in weniger sauren Lösung gearbeitet hatten, wodurch aus ihrem Produkt 1 Mol p-Aminobenzoesäure abgespaltet worden sein konnte. Wir haben deswegen den nach ihrer Angabe angestellten Versuch A nun in neutraler Lösung angestellt (Forrest und Walker machen über die Acidität ihrer Reaktionsmischung keine Angaben).

0,11 g Redukton in 10 ml Wasser gelöst werden mit 0,34 g fester p-Aminobenzoesäure versetzt, die bei 20° fein verrieben wird. Dabei fällt das Reaktionsprodukt als orangerotes Krystallpulver aus, das abfiltriert und mit Wasser, Alkohol und Äther gereinigt wird; Ausbeute 0,263 g. Das Produkt erwies sich identisch mit dem vorher aus essigsaurer Lösung erhaltenen. Auch die Ausbeute stimmt durchaus mit der früher erhaltenen.

Zur weiteren Charakterisierung der nach Verfahren A und B erhaltenen Produkte wurden dieselben mit Natronlauge (0,098 n.) und Phenolphtalein titriert. Verbrauch 2,93 ml. Da der Umschlag wegen der Dunkelgelbfärbung mit Alkali schwer zu sehen ist, wurde noch eine weitere Titration ausgeführt.

26,8 mg Sbst. verbrauchten 2,25 ml Natronlauge.

Als Mittelwert aus beiden Titrationen ergab sich $E_{gef.}$ 108
Für das Mono-p-Aminobenzoes.-Redukton $E_{ber.}$ 104
Für das Di-p-Aminobenzoesäure-Redukton $E_{ber.}$ 163

Die alkalische Titration zeigt also deutlich, dass die Monoverbindung vorliegt.

Titration mit TR-Lösung

0,7 mg Sbst. wurden in 20 ml abs. Alkohol suspendiert und im N_2-Strom mit 0,01 n. TR-Lös. bei 20° titriert. Verbrauch 5,9 ml. Wegen der Schwerlöslichkeit der Subst. in Alkohol erforderte die Titration 1 $^1/_2$ Stunden. In dieser Weise wurde erhalten $E_{gef.} = 113$.

Eine entsprechende Titration in alkalischer Lösung (in der die Subst. sich leicht löst) ergab $E_{gef.} = 115$.

Für die Mono-Verbindung mit freiem Endiol berechnet sich $E_{ber.} = 104$.

Das Ergebnis von Forrest und Walker betr. Bildung einer Redukton-di-p-Aminobenzoesäure können wir nicht erklären.

Kondensation von Redukton mit 5-Amino-Salicylsäure (H. HASSELQUIST und ARMIN GLASER)

Darstellung. 0,23 g Redukton in wenig Wasser gelöst wurden mit einer wässrigen Lösung (75 ml) von 0,96 g 5-Amino-salicylsäure-hydrochlorid (1 : 2) versetzt, das nach der Vorschrift von EULER und HASSELQUIST[1] dargestellt war. Beinahe sofort fällt das Reaktionsprodukt in Form kleiner gelber Nadeln aus. Nach 3 Stunden wurde abfiltriert und der Niederschlag mit Wasser, Alkohol und Äther gewaschen. Ausbeute 0,601 g. Bei 235° färbt sich die Substanz dunkel und bei weiterer Temperaturerhöhung tritt Verkohlung ohne Schmelzen ein.

Alkali-Titration. 22,6 mg Subst. in 10 ml Wasser aufgeschlämmt werden mit 0,098-n NaOH und Phenolphtalein titriert. Verbrauch = 2,01 ml

$E_{\text{gef.}} = 113$

Für Mono-Verbindung $E_{\text{ber.}} = 112$

Für Di-Verbindung $E_{\text{ber.}} = 179$

Hieraus ergibt sich deutlich, dass eine Mono-aminosalicylsäure-Verbindung des Reduktons vorliegt.

Kondensation von Redukton mit 4-Aminosalicylsäure

Darstellung. Dieselbe wurde in analoger Weise wie oben beschrieben ausgeführt. Das Ausgangsmaterial, 4-Aminosalicylsäure, die durch die Untersuchungen von BARRON und LEHMANN bemerkenswert ist, verdanken wir der Chemischen Fabrik FERROSAN, Malmö.

Auch dieses Produkt erwies sich durch die TR-Titration als eine Redukton-Mono-Aminosalicylsäure.

Zur Biochemie der Redukton-Derivate

Auf die Rolle der Redukton-Derivate als Wuchsstoffe und als Antibiotica kommen wir an anderer Stelle zurück. Wie wir schon früher[4] mitteilten, treten Reduktone selbst nur in kleinen Mengen bei Hefengärungen in erheblichen Mengen bei verschiedenen Bakteriengärungen auf. In welchem Grad Triose-Redukton, Ascorbinsäure und andere aliphatische Reduktone an Aminosäuren gebunden sind ist noch nicht bekannt. Dagegen sind die Kombinationen von Redukton mit Pteroylverbindungen bereits Gegenstand eingehender Versuche.

Schliesslich mögen hier noch Phosphorsäure-Derivate des Reduktons erwähnt werden, welche man in ähnlicher Weise wie die Ester organischer Säuren gewinnen kann. Ihre Beteiligung am oxydo-reduktiven Stoffwechsel bleibt noch aufzuklären.

Stockholm, Universität, Institut für organ. chem. Forschung.

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[3] J. LEHMANN, Lancet 1946, I, 15.
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Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

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On the stability of the cyanobenzoic acids in aqueous solution

By Sigvard Wideqvist

The three isomeric cyanobenzoic acids may be prepared from the corresponding aminobenzoic acids according to the reaction of Sandmeyer (1). However, Sandmeyer himself was of the opinion that only the *m*-cyanobenzoic acid could be obtained in this way. Instead of the *p*-cyanobenzoic acid he thought that he had obtained tere-phthalamic acid. He reported that the *p*-cyanobenzoic acid is unstable and changes into tere-phthalamic acid upon crystallization from hot water. However, Kattwinkel and Wolffenstein (2) showed that the cyanoacid can be prepared by the Sandmeyer method and can be recrystallized from hot water. In one experiment they found that the cyanoacid remained unchanged after two hours boiling with water.

Later *p*-cyanobenzoic acid was prepared by Valby and Lucas (3) who determined its dissociation constant.

In order to study its stability against hydrolysis, the author kept a water solution of the acid in a conductivity cell at 25° for 48 hours. No change in the conductivity was observed during this time.

m-Cyanobenzoic is, like the *p*-cyanobenzoic acid, not hydrolyzed in aqueous solution.

When Sandmeyer tried to prepare *o*-cyanobenzoic acid from anthranilic acid, he obtained phthalimide as reaction product. The same product was obtained, when Sandmeyer's experiment was repeated by Tiemann and Müller (4). Sandmeyer assumed that the cyanoacid had been hydrolyzed to phthalamic acid which, on dehydration, yielded phthalimide. *o*-Cyanobenzoic acid has, however, been obtained in poor yield in several ways. A summary of the methods of preparation is given by Scheiber (5). The best method is, as found by the author, to apply the method of Sandmeyer to ethyl anthranilate, whereby ethyl *o*-cyanobenzoate is obtained. On saponification of the ester with an equivalent amount of alkali, *o*-cyanobenzoic acid is obtained in a good yield.

In agreement with the observations of Sandmeyer *o*-cyanobenzoic acid is easily hydrolyzed to phthalamic acid which is very easily converted into phthalimide. *o*-Cyanobenzoic acid itself is readily converted into phthalimide when heated over the melting point, as reported by Scheiber (5) and others.

The dissociation constant of *o*-cyanobenzoic acid was determined by Scheiber (5), who reported that the dissociation constant was strongly dependent on the concentration of the acid and gave the following values ($\Lambda_0 = 372$):

c	$K \cdot 10^4$
0.001000	7.00
0.000500	5.63
0.000250	4.29

SCHEIBER assumed that the *o*-cyanobenzoic acid was in equilibrium with some tautomeric, non acid or slightly acid form which was responsible for the decreasing of the ionization constant, but he did not believe that it was dependent on hydrolysis of the cyano group. However, there is no doubt that the curious behaviour of the acid is actually dependent on hydrolysis of this group. When the conductivity of *o*-cyanobenzoic acid was studied by the author, it was found that the conductivity at first rapidly decreased, then passed through a minimum and finally increased. *o*-Cyanobenzoic acid is hydrolyzed to the weaker phthalamic acid, and the phthalamic acid is then hydrolyzed to ammonium hydrogen phthalate which is a strong electrolyte. When the conductivity solution was prepared, it was necessary to dissolve the acid in a small quantity of acetone, after which water was added. *o*-Cyanobenzoic acid is in fact very slightly soluble in water and goes very slowly into solution, as it is strongly hygrophobe. When no acetone is added, the time for dissolution is several hours, by which time the cyanoacid is largely hydrolyzed.

Phthalamic acid, the product of hydrolysis of *o*-cyanobenzoic acid, was prepared by ASCHAN (6) by the action of 15 per cent potassium hydroxide on phthalimide. Phthalamic acid is also conveniently obtained from phthalic anhydride which, when treated with concentrated ammonia, gives the ammonium salt of phthalamic acid. The dissociation constant of phthalamic acid was determined by OSTWALD (7) who, curiously enough, does not seem to have observed that the acid rapidly changed its conductivity. At first the conductivity decreases, passes through a minimum and then rapidly increases.

Experimental

The preperation of material

Ethyl o-cyanobenzoate. Ethyl anthranilate hydrochloride, 20.2 g, was dissolved in a mixture of 20 ml of water and 10 ml of concentrated hydrochloric acid and 100 g of ice was added. The solution was diazotized with 8 g of sodium nitrite in 20 ml of water.

Twentytwo grams of potassium cyanide and 15 g of cuprous cyanide were dissolved in 45 ml of water, and 50 ml benzene was poured on the surface. The solution was chilled to 0–5°, and the neutralized solution of the diazonium salt was slowly added under rapid stirring. After the addition the solution was kept at room temperature for one hour. The benzene layer was then separated and the water solution was extracted with ether. The combined ether and benzene solutions were dried with anhydrous magnesium sulfate. On evaporation of the solvent, ethyl *o*-cyanoacetate was obtained as a brownish crystalline mass. Yield 12 g (68.5 %). It was recrystallized from dilute alcohol.

o-Cyanobenzoic acid. Sodium, 0.95 g, was dissolved in a mixture of 20 ml of absolute alcohol and 0.74 ml of water. The warm solution was rapidly mixed with a solution of 7.2 g of ethyl *o*-cyanobenzoate in 25 ml of warm absolute alcohol. A sodium salt separated almost immediately. The mixture was kept at room temperature overnight. The salt was then filtered off by suction and washed with absolute alcohol. Yield 6.8 g. The sodium salt was dissolved in water, and 5 ml of concentrated hydrochloric acid was added, whereby *o*-cyanobenzoic acid separated as a thick mass which was filtered off with suction. Yield 5 g. The acid was recrystallized from acetone. M. p. 190°.

Anal. Subst., 24.07 mg: CO_2, 57.61 mg; H_2O, 7.67 mg.
Calcd. for $C_8H_5O_2N$ C, 65.28; H, 3.43.
Found C, 65.27; H, 3.57.

p-Cyanobenzoic acid was prepared from *p*-aminobenzoic acid according to SANDMEYER (1). The directions given by VALBY and LUCAS (3) were followed. The product was first repeatedly recrystallized from hot water under addition of Norite. As the acid was still impure, it was dissolved in boiling diethyl carbonate; a great deal of dark colored impurities were then left undissolved and were filtered off. On cooling the solution, a slightly yellowish product separated. Finally it was recrystallized from hot 25 per cent alcohol. M. p. 212–214°.

m-Cyanobenzoic acid was prepared from *m*-aminobenzoic acid according to SANDMEYER, but nickel cyanide was used instead of cuprous cyanide. The product was recrystallized several times from hot water under addition of Norite. Finally it was recrystallized from hot 50 per cent acetone. M. p. 217–218°.

Phthalamic acid was prepared by dissolving phthalic anhydride in about ten times its weight of concentrated ammonia. The solution was left at room temperature for 24 hours. The excess of ammonia was evaporated in vacuo. The solution was filtered and then acidified with concentrated hydrochloric acid, whereupon phthalamic acid slowly separated. The acid was filtered off and washed with water. It was then dissolved in dilute sodium hydroxide and the solution was acidified with hydrochloric acid. Phthalamic acid separated as beautiful crystals. The crystals were washed with water and dried in the air. M. p. 148°.

The measurements

The dissociation constant of o-cyanobenzoic acid. Samples of the acid were dissolved in 0.8 ml of acetone, "Kahlbaum, Aus der Bisulfitverbindung." Water was then added to make the volume 100 ml. The solution was rapidly transferred to a conductivity cell which was immersed in a thermostat at 25° C. The conductivity was determined each third minute during one to two hours and was then extrapolated to zero time. The conductivity decreased about 5 per cent in one hour.

For the equivalent conductance at zero concentration the value 377 reciprocal ohms, given by VALBY and LUCAS for the *p*-cyanobenzoic acid, was adopted. (Table I.)

Phthalamic acid. In conformity to the *o*-cyanobenzoic acid, the phthalamic acid rapidly changes its conductivity because of hydrolysis. At first the conductivity decreases; then it passes through a minimum, and finally it increases. The conductivity was determined in the same manner as for *o*-cyanobenzoic acid, but the solvent was pure water; no acetone was added. A typical run of measurements is given in Table II. The results of the determination of the dissociation constant are given in Table III.

Table I.

The dissociation constant of *o*-cyanobenzoic acid.

25° C; $\Lambda_0 = 377$

c	Λ_c	α	$K \cdot 10^4$
0.002556	154.1	0.4088	7.22
0.001164	202.9	0.5382	7.30
0.000598	246.2	0.6531	7.35

Mean value $7.29 \cdot 10^{-4}$

Table II.

The conductivity of phthalamic acid.

$c = 0.006345$; 25° C; t in minutes.

t	$1000\,\kappa$	t	$1000\,\kappa$
0	(0.36805)	81	0.36469
12	0.36711	84	469
15	687	87	475
18	662	90	481
21	664	96	493
27	602	99	499
30	583	105	517
45	511	117	571
57	475	120	580
60	475	168	901
63	469	171	926
66	463	174	950
69	463	177	975
72	463	180	0.37000
75	463	192	112
78	463	195	143

Table III.

The dissociation constant of phthalamic acid.

25° C; $\Lambda_0 = 375$.

c	Λ_c	α	$K \cdot 10^4$
0.006345	58.01	0.1546	1.79
0.003196	78.07	0.2081	1.75
0.001586	107.7	0.2817	1.73

Mean value $1.76 \cdot 10^{-4}$

p-Cyanobenzoic acid. The conductivity for a 0.001765-m solution of *p*-cyanobenzoic acid was observed during 48 hours at 25° C. The following values were obtained:

Time (hours)	0	28	48
1000 $\varkappa$	0.21955	0.21951	0.21959

From this the value $2.9 \cdot 10^{-4}$ for the dissociation constant of *p*-cyanobenzoic acid is obtained. VALBY and LUCAS reported the value $3.1 \cdot 10^{-4}$.

m-Cyanobenzoic acid. The conductivity for a 0.001854-m solution of *m*-cyanobenzoic acid was observed at 25° C. No appreciable change in the conductivity was observed during 24 hours. The specific conductance was 0.21254 reciprocal ohms. From this the value $2.5 \cdot 10^{-4}$ for the dissociation constant of *m*-cyanobenzoic acid is obtained. According to KILPATRICK and EANES (8) the ratio between the dissociation constants of *m*-cyanobenzoic acid and *p*-cyanobenzoic acid is 331/448. But the dissociation constant of *p*-cyanobenzoic acid was $3.1 \cdot 10^{-4}$. From this the value $2.3 \cdot 10^{-4}$ for the dissociation constant of *m*-cyanobenzoic acid is obtained.

Acknowledgment. The author is indebted to Fil. mag. GÖSTA RUDSTAM and Fil. stud. ÅKE SILJEHOLM for the preparation of the crude products of *p*- and *m*-cyanobenzoic acid.

SUMMARY

The three isomeric cyanobenzoic acids have been prepared and their conductivity in water solution studied at 25° C. It was found that the conductivity of *m*- and *p*-cyanobenzoic acid did not change with time, but the conductivity of *o*-cyanobenzoic acid rapidly decreased dependent on hydrolysis of the cyano group. On hydrolysis, *o*-cyanobenzoic acid gives phthalamic acid which is readily hydrolyzed to ammonium hydrogen phthalate. The behaviour of *o*-cyanobenzoic acid in aqueous solution is in conformity with that of β-cyanosubstituted fatty acids, *e.g.* β-phenyl-β-cyanopropionic acid which is hydrolyzed to phenylsuccinamic acid in water solution at room temperature. The amic acid is then hydrolyzed to ammonium hydrogen phenyl-succinate. Carboxylic acids with a cyano group in α- or γ-position to the carboxy group are, however, not hydrolyzed to the corresponding amic acids in water solution at room temperature.

Laboratory of Organic Chemistry, University of Uppsala.

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The phase systems fluorobenzoic-chlorobenzoic acids and fluorobenzoic-hydroxybenzoic acids

By JERKER PORATH and GÖRAN CLAESON

With 7 figures in the text

Phase diagrams for mixtures of hydroxy compounds and analogous chlorine compounds often exhibit simple eutectic points. The difference in molecular volume and polar character are probably the main reason why solid solutions between compounds of this kind are not easily formed. It seems very probable that corresponding fluorine compounds occupy an intermediary position. Chlorine, bromine and iodine compounds are, as a rule, isomorphous, and even if fluorine can be expected to occupy a particular position in relation to the other halides, a certain similarity between analogous fluorine and chlorine compounds should be revealed in the phase diagrams. On the other hand the fluorine atom and the hydroxyl group have rather similar radii and, moreover, they are both strongly electronegative.

In order to find out whether the assumption above is correct, we have chosen substituted benzoic acids, partly because fluorobenzoic acids were most readily available among fluorine compounds and have suitable physical properties, and partly because this research will be related in a natural way to the works of LETTRÉ and his collaborators concerning the isomorphism of substituted benzoic acids (1).

LETTRÉ found that the different chlorobenzoic acids formed continuous series of mixed crystals with the corresponding bromobenzoic acids. The phase diagrams of the hydroxybenzoic acids and chlorosubstituted benzoic acids exhibited, however, simple eutectic points.

As a preliminary survey, we studied the powder photographs of p-hydroxybenzoic acid and all the p-halobenzoic acids. The corresponding interference lines of the chloro-, bromo- and iodobenzoic acids are plainly visible. The powder photographs of both p-fluorobenzoic acid and hydroxybenzoic acid differ from those of the rest of the halobenzoic acids. Isomorphism exists only among the acids of chlorine, bromine and iodine which LETTRÉ found to give phase diagrams of the type I of ROOZEBOOM.

Fig. 1 and 2 representing the phase diagrams of p-fluorobenzoic acid mixed with p-hydroxy and p-chlorobenzoic acid respectively are of the type V of ROOZEBOOM. The ability of p-fluorobenzoic acid to replace the p-chlorobenzoic in the crystal lattice of the latter is particularly obvious. Powder photographs of mixtures of p-fluoro- and p-hydroxybenzoic acid show that the mutual miscibility in the solid state is just slightly less at room temperature.

Fig. 3 and 4 show that the *meta* acids give diagrams similar to those of the *para* acids.

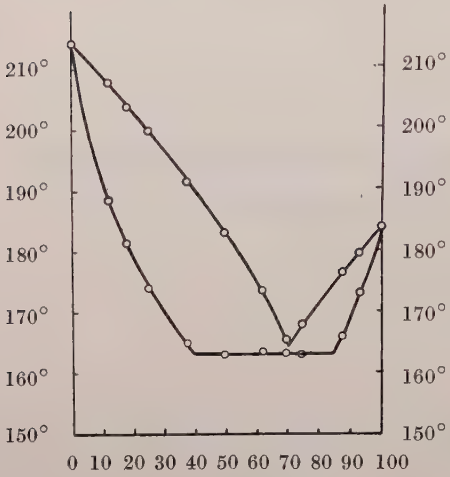

Fig. 1. p-fluorobenzoic acid + p-hydroxybenzoic acid.

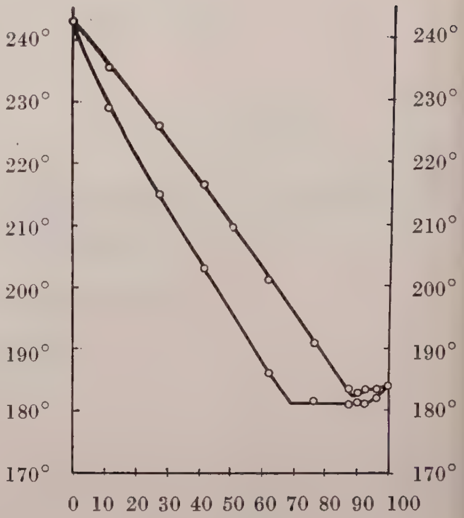

Fig. 2. p-fluorobenzoic acid + p-chlorobenzoic acid.

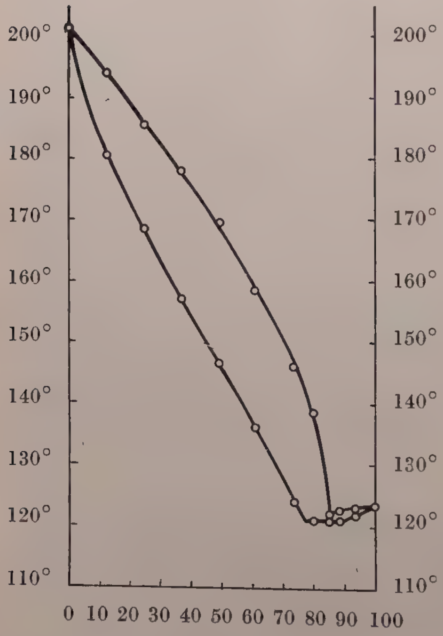

Fig. 3. m-fluorobenzoic acid + m-hydroxybenzoic acid.

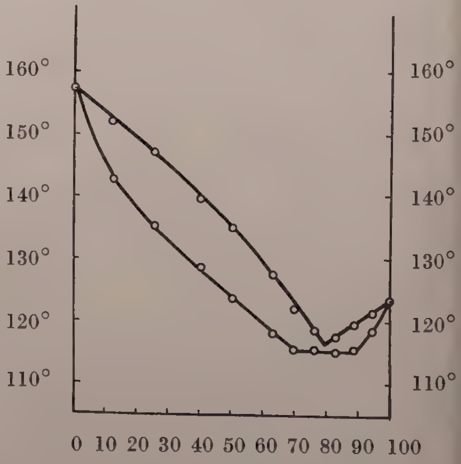

Fig. 4. m-fluorobenzoic acid + m-chlorobenzoic acid.

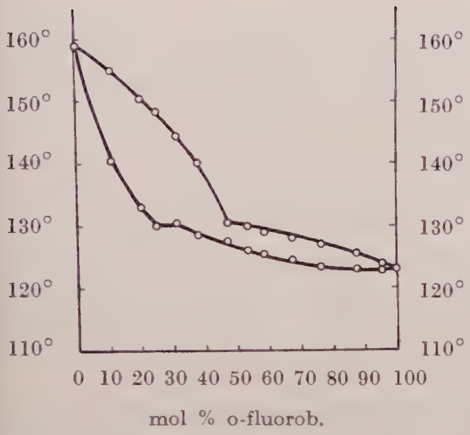

Fig. 5. o-fluorobenzoic acid + o-hydroxybenzoic acid.

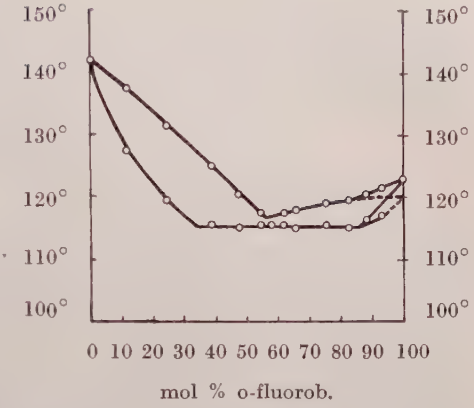

Fig. 6. o-fluorobenzoic acid + o-chlorobenzoic acid.

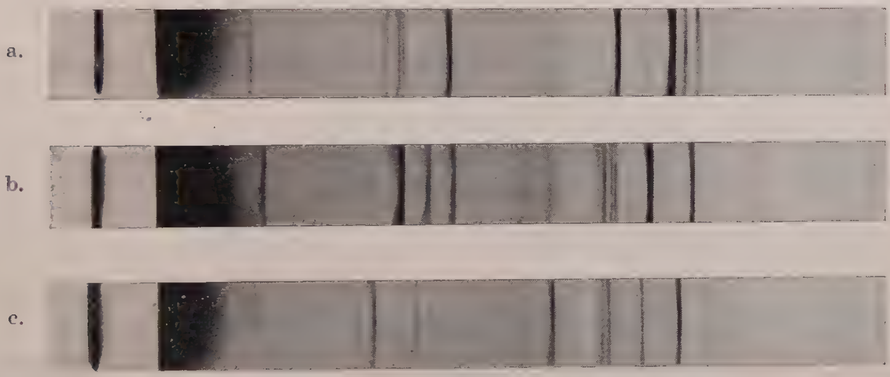

Fig. 7.

a. o-fluorobenzoic acid.
b. 75 % o-fluorobenzoic acid + 25 % o-chlorobenzoic acid.
c. o-chlorobenzoic acid.

The diagrams of the *ortho* acids differ slightly from the preceding, both exhibiting peritectic points. Fig. 5, the system o-fluorobenzoic acid—o-hydroxybenzoic (salicylic) acid, is of the type IV of ROOZEBOOM with pronounced mutual miscibility. Powder photographs of a mixture of 50 % of each component showed only the interference lines of the fluorobenzoic acid. The diagram o-fluorobenzoic acid—o-chlorobenzoic acid (fig. 6) shows that, except for the stable form melting at 123°, an unstable o-fluorobenzoic acid with its melting point at about 120° can evidently exist in a stabilized form in the interval 57—83 % o-fluorobenzoic acid. Efforts to produce this modification failed. A powder photograph (fig. 7 b) of a mixture of 75 % o-fluorobenzoic acid and 25 % o-chlorobenzoic acid indicates, however, that the lattice is of another type than that of the pure components (fig. 7 a and 7 c), and the liquidus curve indicates that it cannot depend on the formation of a molecular compound with incongruent melting point.

All of the diagrams show that the corresponding compounds form solid solutions with each other to a limited extent. Consequently, the fluorobenzoic acids in this respect occupy a position intermediate between the chlorobenzoic and the hydroxybenzoic acids.

Experimental

The preparation of the acids used for the melting point determinations.

p-hydroxybenzoic acid "rein" from Schuckart was purified. The original material, which contained coloured substances, was shaken with a mixture of water, acetone and ether. Thereby the water-acetone phase became blue. The ether phase was treated with sodium hydroxide solution. The colourless waterphase was separated, acidified with sulphuric acid and extracted with ether.

The acid obtained from the ether was recrystallized from water. The recrystallization was repeated until a constant melting point was reached. On one occasion unstable crystals were formed; the acid crystallized out in long colourless needles, which, after a few minutes, were transformed into the ordinary crystal form: rhombic plates. Because the acid crystallizes as a monohydrate, it was dried over concentrated sulphuric acid before each melting point determination. M. p. 214.5°.

p-fluorobenzoic acid "Eastman" was recrystallized twice from water and twice from 30 % ethanol. In the latter case the crystals contained ethanol that was removed in an oven. The acid can also crystallize with acetone. The dried acid melted at 184°.

p-chlorobenzoic acid "Eastman" was recrystallized four times from benzene yielding a product melting at 243°.

m-hydroxybenzoic acid "reinst" from Schuckart, was recrystallized once from ethyl acetate and three times from water. M. p. 201.5°.

m-fluorobenzoic acid was obtained from m-fluorotoluene on oxidation with potassium permanganate. The two following methods were used:

1) The procedure used by SLOTHOUWER (2). Yield 52 % (SLOTHOUWER obtained 53 %). Four recrystallizations from water yielded an acid with m. p. 123.5°.

2) 18 g m-fluorotoluene and a solution containing 60 g potassium permanganate in 700 ml water were poured into a 2 liter flask. The content was refluxed 15 hours on an oil bath and then distilled. The unchanged fluorotoluene distilled together with the water vapour. When the water drops became clear the distillation was finished. 7 g m-fluorotoluene was recovered from the distillate. The residue was acidified with dilute sulphuric acid and a current of sulphur dioxide was passed through the solution until all the manganese dioxide had been reduced. The contents were evaporated to 350 ml. The manganous salt that had settled was dissolved in sulphuric acid. On acidifying the m-fluorobenzoic acid precipitated. Treatment with ether followed by evaporation yielded 9 g m-fluorobenzoic acid (64 %).

0.1818 g acid: 13.99 ml 0.0930-N NaOH
E. W. calc. 140.1; found 139.7

m-chlorobenzoic acid was obtained from m-chlorotoluene by a method analogous to 2 above. M. p. 157.5°.

o-hydroxybenzoic (*salicylic*) *acid.* Baker's "Analyzed," was recrystallized once from water. M. p. 159°.

Table I.

p-fluorobenzoic acid and p-hydroxybenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		214.5
11.6	188.5	208.0
17.7	181.5	204.0
24.6	174.0	200.0
37.2	165.0	191.5
49.4	163.0	183.5
61.8	163.5	173.5
69.4	163.0	165.5
74.5	163.0	168.0
87.6	166.0	176.5
93.1	173.0	179.5
100.0		184.0

Table II.

p-fluorobenzoic acid and p-chlorobenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		243.0
11.2	229.0	235.5
27.5	215.0	226.0
41.7	203.0	216.5
50.9	191.5	209.5
62.2	186.0	201.0
76.4	181.5	191.0
87.8	181.0	183.5
90.2	181.5	183.0
92.6	181.0	183.5
96.3	182.0	183.5
100.0		184.0

o-*fluorobenzoic acid* was obtained by a method similar to that used for synthesizing m-fluorobenzoic acid. 18 g o-fluorotoluene yielded 9.1 g acid. 8 g o-fluorotoluene was recovered. Yield 72 %. The acid was recrystallized from water until constant melting point 183°.

0.1838 g acid: 14.09 ml 0.0930-*N* NaOH
E. W. calc. 140.1; found 140.3

o-chlorobenzoic acid was prepared from anthranilic acid and recrystallized several times from dilute alcohol. M. p. 142°.

Table III.

m-fluorobenzoic acid and m-hydroxybenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		201.5
12.4	180.5	194.0
24.9	168.5	185.5
37.0	157.0	178.0
49.7	146.5	169.5
61.0	136.0	158.5
73.8	124.0	146.0
80.1	121.0	138.5
85.2	121.5	122.0
88.6	121.0	122.5
93.3	122.0	123.0
100.0		123.5

Table IV.

m-fluorobenzoic acid and m-chlorobenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		157.5
12.5	142.5	152.0
25.7	135.0	147.0
40.4	128.5	139.5
50.6	123.5	135.0
63.7	118.0	127.5
70.1	115.5	122.0
76.6	115.5	118.5
83.1	115.0	117.5
89.0	115.5	119.5
94.9	118.5	121.5
100.0		123.5

X-ray photographs.

Powder photographs were taken with CuK$_\alpha$-radiation, a monochromator of the Guinier type (3) being used. Time of exposure two hours.

The preparations to be compared were obtained from evaporation of the same kind of solvent.

Phase diagrams.

Melting point determinations were performed by Rheinboldts method (4) with Friedrichs' melting point apparatus (5). Both components were weighed into a test tube, mixed, melted together, and cooled. The contents were then pulverized and brought into melting point capillary tubes. In case sublimation might occur, the tube was melted shut just above the sample. A calibrated thermometer was used.

Table V.

o-fluorobenzoic acid and o-hydroxybenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		159.0
10.8	140.5	155.0
20.0	133.0	150.5
25.1	130.0	148.5
31.2	130.5	144.5
38.0	128.5	140.0
40.2	127.5	138.0
47.1	127.5	130.5
53.2	126.0	130.0
58.5	125.5	129.0
67.2	124.5	128.0
76.1	123.5	127.0
87.7	123.0	125.5
95.6	123.0	124.0
100.0		123.0

Table VI.

o-fluorobenzoic acid and o-chlorobenzoic acid.

mol % fluorobenzoic acid	Thaw point	Melting point
0.0		142.0
11.7	127.5	137.5
24.3	119.5	131.5
38.9	115.5	125.0
47.5	115.0	120.5
54.6	115.5	117.5
57.9	100.0	115.5
61.9	115.5	117.5
65.6	115.0	118.0
75.4	115.5	119.0
82.6	115.0	119.5
88.3	116.5	120.5
93.2	117.0	121.5
100.0		123.0

SUMMARY

Phase diagrams and powder photographs have shown that the three fluorobenzoic acids form solid solutions to a limited extent with both the corresponding chlorine and hydroxy acids. In this respect, the fluorine compounds thus occupy an intermediate position between the chlorine and the hydroxy compounds.

m-fluoro- and o-fluorobenzoic acid have been prepared by a somewhat modified method from the corresponding fluorotoluenes with respective yields of 64 % and 72 %.

The authors are indebted to Professor ARNE FREDGA for specimens of material as well as for valuable advice and discussions.

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Organic Department, Chemical Institute, University of Uppsala, Sweden December 1949.

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Communicated 10 May by ARNE WESTGREN and PERCY QUENSEL

The crystal structure of Diaboleite, $Pb_2Cu(OH)_4Cl_2$

By ANDERS BYSTRÖM and KARL-AXEL WILHELMI

With 2 figures in the text

Introduction

In the course of an investigation of lead oxides and lead fluorides by one of us it was found that the arrangement of the oxygen and the fluorine atoms around the bivalent lead atoms differed very much from compound to compound, the coordination numbers being 3, 4, 6, 8 and 9 in the structures investigated up till now (1, 2). Because of the low scattering factors of the oxygen and the fluorine atoms compared with the lead atom, it is often very difficult to prove the correctness of the suggested positions for the negative atoms. Only in two cases do the x-ray reflections prove the validity of the arrangement (Tetragonal PbO: DICKINSON and FRIAUF (5), MOORE and PAULING (6), BYSTRÖM (3); Pb_3O_4: BYSTRÖM and WESTGREN (4), BYSTRÖM (1)). In one or two other cases, the arrangement of the lead atoms and the cell dimensions suggest that the compounds belong to isostructural series (the two modifications of PbF_2 (2)) and in that case the assumption that the positions of the negative atoms in the lead compounds are the same as for the other members in the series is justified, but not absolutely proved. However, in still other cases neither conclusions from the observed intensities nor comparisons with isomorphous compounds are possible, and often the symmetry of the crystals would allow several different arrangements of the atoms. Which of these arrangements is valid must then be decided from comparisons with the interatomic distances and coordination figures in structures already known. It is, then, of interest to increase the number of definitively established structures. Obviously a small number of atoms in the unit cell and a high symmetry increase the possibility of a complete structure determination.

Determination of the atomic arrangement

In the case of diaboleite, both of these conditions exist. The unit cell has been determined by PALACHE (7) to be tetragonal with $a = 5.83$ Å and $c = 5.42$ Å with a cell content of $Pb_2Cu(OH)_4Cl_2$. The Laue symmetry derived from the Weissenberg photographs is $4/mmm$, and as no systematic absences are noted among the reflexions and morphological evidence shows that the 4-fold axis is hemimorphic, PALACHE concludes that the space group is $P4mm$. PALACHE also noted that the crystals have a perfect and brilliant cleavage parallel to $\{0\,0\,1\}$ and that the hardness is $2\frac{1}{2}$.

Our investigation[1] confirmed PALACHE's findings in every respect. Rotation photographs around $[1\,0\,0]$ and Weissenberg photographs of the $0\,k\,l$, $1\,k\,l$ and $2\,k\,l$ reflexions showed the presence of reflexions of all types and as a Laue photograph with the beam perpendicular to $(0\,0\,1)$ showed the Laue symmetry $D_{4h} - 4/mm$, the possible space groups are D^1_{4h}, D^1_4, C^1_{4v}, D^1_{2d}, D^5_{2d}. A remarkable feature in the intensity distribution is that the reflexions with $h + k = 2n + 1$ are very weak or absent, indicating that the two lead atoms, which have by far the dominating scattering power among the atoms, are placed in a c-face centered position. In D^1_{4h} and D^1_4 the Pb, Cu, and Cl-atoms must be situated in $z = 0$ or $z = \frac{1}{2}$ (Cl — Cl distances of $\frac{c}{2} = 2.7$ Å which would result in $2\,(g)$ and $2\,(h)$ can safely be ruled out as impossible), and this is not in accordance with the observed intensities of the $0\,0\,l$ reflexions. In D^5_{2d} there are three ways to place the lead atoms:

1) $2\,(g)$: $0\,\frac{1}{2}\,z$; $\frac{1}{2}\,0\,\bar{z}$ with $z = 0$ or $\frac{1}{2}$,

2) $1\,(a)$: $0\,0\,0 + 1\,(b)$: $\frac{1}{2}\,\frac{1}{2}\,0$,

3) $1\,(c)$: $\frac{1}{2}\,\frac{1}{2}\,\frac{1}{2} + 1\,(d)$: $0\,0\,\frac{1}{2}$.

However, in all these cases the Cu and Cl atoms must be placed in $z = 0$ or $\frac{1}{2}$, but this is ruled out by the $0\,0\,l$ intensities. As D^1_{2d} gives no further alternative to place the atoms, C^1_{4v} is the only possible space group.

In C^1_{4v} the lead atoms can be placed either in $1\,(a)$: $0\,0\,z_1$, $+\,1\,(b)$: $\frac{1}{2}\,\frac{1}{2}\,z_2$ with $z_1 = z_2 = 0$ or $\frac{1}{2}$, or in $2\,(c)$: $\frac{1}{2}\,0\,z$, $0\,\frac{1}{2}\,z$. With the former arrangement the arrangement of the Cu and Cl-atoms must be one of the following:

1) 2 Cl in $1\,(a)$: $0\,0\,z_3 + 1\,(b)$: $0\,0\,z_4$ with $z_3 \sim z_4 \sim \frac{1}{2}$ or 0. The Cu atom must then be placed in a half-filled position $2\,(c)$ but then the intensities of the reflexions with $h + k = 2n + 1$ cannot be explained.

2) The Cl atoms in $2\,(c)$. The Cu atom in $1\,(a)$, $1\,(b)$ or in an incompletely filled position $2\,(c)$. The reflexions with $h + k = 2n + 1$ exclude this alternative.

It then only remains to place the Pb atoms in $2\,(c)$. The z parameter of the lead atoms can be arbitrarily fixed as 0. The Cl atoms can then be placed in $2\,(c)$ but in that case $z_{Cl} \sim \frac{1}{2}$, which is contradicted by the intensities of the reflexions $0\,0\,l$. As the arrangement 2 Cl in $1\,(a) + 1\,(b)$ and Cu in $2\,(c)$ is also impossible, for the same reason, only the alternative 2 Cl in $1\,(a) + 1\,(b)$ and Cu in $1\,(a)$ remains.

The oxygen atoms must be placed in a fourfold position because a combination of one and/or twofold positions leads to a superposition of three or more atoms in the c direction, which is impossible for spatial reasons. Of the fourfold positions, $4\,(e)$ and $4\,(f)$ give O — O distances of 2.4 Å or less, and

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[1] Professor F. WICKMAN kindly provided us with a mineral specimen of diaboleite (Mammoth Mine, Tiger, Arizona) out of the collections of the Swedish Museum of Natural History, Department of Mineralogy, Stockholm (specimen number 470234).

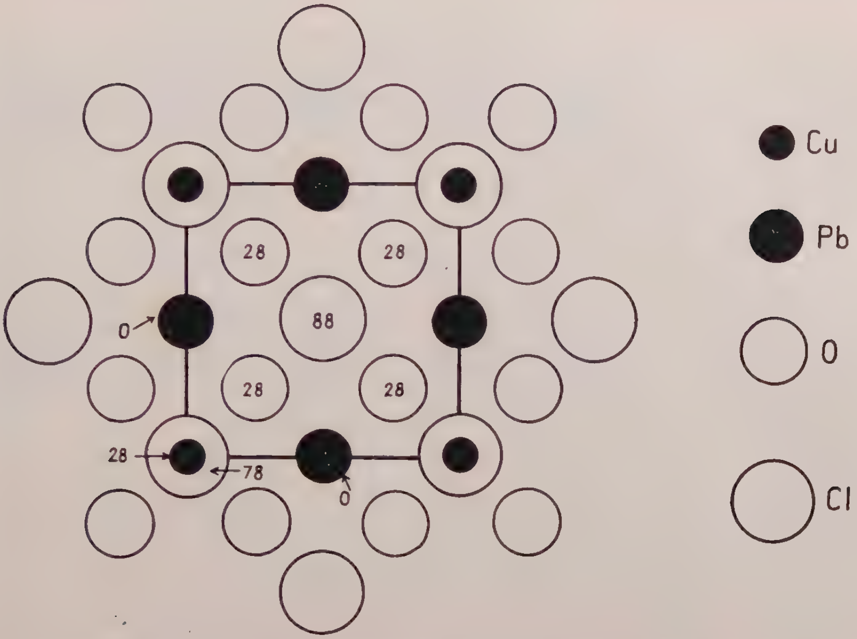

Fig. 1. Diaboleite, $Pb_2Cu(OH)_4Cl_2$. Projection on (0 0 1). The figures denote the height of the atoms in percentages of c.

distances as short as these are hardly possible in a structure with large atoms like Cu and Pb.

The following arrangement is thus obtained:

2 Pb in 2 (c): $\frac{1}{2}\,0\,z_1;\ 0\,\frac{1}{2}\,z_1,\ 2\pi z_1 = 0,$
1 Cu 1 (a): $0\,0\,z_2,$
1 Cl_1 1 (a): $0\,0\,z_3,$
1 Cl_2 1 (b): $\frac{1}{2}\,\frac{1}{2}\,z_4,$
4 O 4 (d): $x_5\,x_5\,z_5;\ x_5\,\bar{x}_5\,z_5;\ \bar{x}_5\,x_5\,z_5;\ \bar{x}_5\,\bar{x}_5\,z_5.$

Because of the low observed value of I (0 0 2) compared with I (0 0 3), z_2 must be ~ 0.25. Then z_3 must for spatial reasons be ~ -0.25. If 2.5 Å is fixed as a reasonable lower limit for the O — O distances, then $0.22 < x_5 < 0.28$ and to obtain approximately normal O — Cl distances, the parameter z_5 must be ~ 0.25. Obviously, because of the O — Cl distances, $z_5 - z_4$ must be ~ 0.5. Since the lead atoms have no influence on the reflexions $h\,k\,l$ with $h + k = 2\,n + 1$ and since the influence of the oxygen atoms on the reflexions $1\,0\,l$ is 0 or very small, the intensities of these reflexions were used to determine the parameters $z_4 - z_2$ and $z_3 - z_2$. Then from the reflexions $0\,0\,l$ the other z-parameters could be determined. Because of strong absorption effects the reflexions at low glancing angles were considerably weaker than expected. It seems that the effect of the absorption and that of the Lp-factor cancel out and therefor in table 1

Table 1

Diaboleite, $Pb_2Cu(OH)_4Cl_2$. Comparison between observed and calculated intensities. Cu-*K* radiation.

h k l	I$_{\text{obs.}}$	F$^2_{\text{calc.}}$
0 0 1	v.st.	27
0 0 2	st.	7.9
0 0 3	v.st.	16
0 0 4	v.st.	17
0 0 5	st.	6.9
0 0 6	st.	5.2
0 0 7	v.st.	10
0 1 0	v.w.$^-$	0.7
0 2 0	m.$^+$	44
0 3 0	—	0.4
0 4 0	m.	26
0 5 0	—	0.2
0 6 0	w.	16
0 1 1	v.w.$^-$	0.6
0 2 1	m.	19
0 3 1	—	0.3
0 4 1	w.	13
0 5 1	—	0.2
0 6 1	w.	9.3
0 1 2	v.w.	0.9
0 2 2	m.	7.1
0 3 2	v.w.	0.6
0 4 2	w.	5.4
0 5 2	—	0.4
0 6 2	w.	4.0
0 1 3	—	0.1
0 2 3	st.	15
0 3 3	—	0.1
0 4 3	m.	11
0 5 3	—	0.1
0 6 3	m.	7.7
0 1 4	v.w.	1.0
0 2 4	st.	15
0 3 4	v.w.$^-$	0.8
0 4 4	st.	12
0 5 4	v.w.	0.6
0 6 4	st.	9.3
0 1 5	—	0.0
0 2 5	m.	4.3
0 3 5	—	0.0
0 4 5	st.	5.1
0 5 5	—	0.0
0 1 6	v.w.	0.6
0 2 6	st.	5.2
0 3 6	v.w.$^-$	0.6
1 1 0	m.	9.4
1 2 0	—	0.5
1 3 0	m.$^-$	6.8
1 4 0	—	0.3
1 5 0	w.	4.6
1 6 0	—	0.2
1 7 0	w.	3.2
1 1 1	m.$^+$	19
1 2 1	—	0.4
1 3 1	m.	13
1 4 1	—	0.2
1 5 1	w.$^+$	8.7
1 6 1	—	0.2
1 7 1	m.$^+$	6.3
1 1 2	st.	26
1 2 2	v.w.$^-$	0.7
1 3 2	m.	19
1 4 2	—	0.5
1 5 2	m.	13
1 6 2	—	0.3
1 7 2	st.	9.4
1 1 3	st.	16
1 2 3	—	0.1
1 3 3	m.$^-$	11
1 4 3	—	0.1
1 5 3	w.$^+$	8.4
1 6 3	—	0.1
1 1 4	m.$^+$	9.5
1 2 4	v.w.$^-$	0.9
1 3 4	m.	7.9
1 4 4	v.w.$^-$	0.6
1 5 4	m.	5.9
1 6 4	v.w.$^-$	0.5
1 1 5	st.	11
1 2 5	—	0.0
1 3 5	st.	9.7
1 4 5	—	0.0
1 5 5	st.	6.8
1 1 6	m.$^+$	9.1
1 2 6	v.w.$^-$	0.5
1 3 6	st.	8.2
2 2 0	m.	34
2 3 0	—	0.3
2 4 0	m.$^-$	13
2 5 0	—	0.2
2 6 0	m.	15
2 2 1	m.$^-$	16
2 3 1	—	0.3
2 4 1	w.	12
2 5 1	—	0.2
2 6 1	w.	7.9
2 2 2	w.	6.4
2 3 2	—	0.5
2 4 2	w.	8.2
2 5 2	—	0.4
2 6 2	v.w.	3.6
2 2 3	m.$^+$	13
2 3 3	—	0.1
2 4 3	w.$^+$	7.6
2 5 3	—	0.1
2 6 3	m.	7.4
2 2 4	st.	14
2 3 4	v.w.$^-$	0.7
2 4 4	m.	7.8
2 5 4	—	0.5
2 2 5	m.	5.1
2 3 5	—	0.0
2 4 5	m.	6.4
2 2 6	st.	5.1
2 3 6	—	0.5

the observed intensities are compared with the calculated F^2-values. The photographs also show that reflexions 0 *k l* with low *l*-values (especially 0 *k* 0) show up with considerably weaker intensities than expected from the F^2-values. This is due to the habit of the crystals. A crystal much smaller than that for which the intensities in Table 1 are registered showed this effect to a lesser degree, as will be seen from the following comparison:

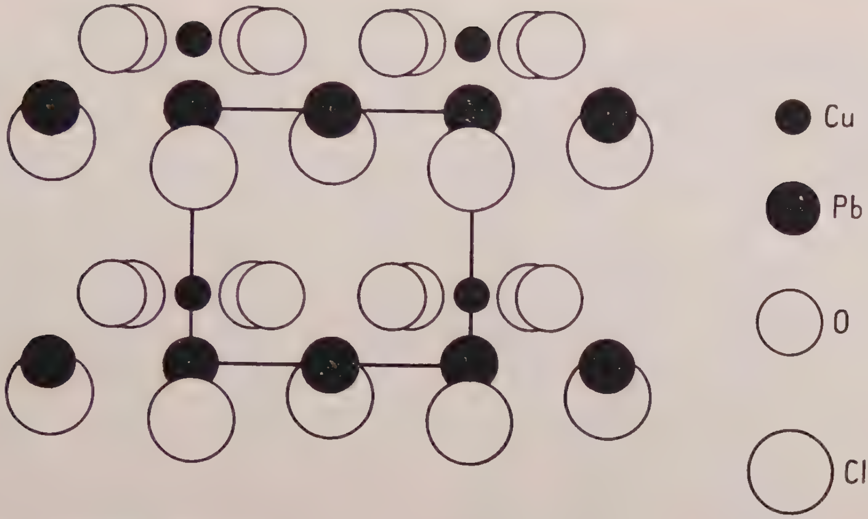

Fig. 2. Diaboleite, $Pb_2Cu(OH)_4Cl_2$. Projection on (0 1 0). Superimposed oxygen atoms are symmetrically displaced. Compare Fig. 1.

	020	021	022	023	024	025	026
$I_{obs.}$	v. st.	st.	m.	m.	m.	m—.	m.
$F^2_{calc.}$	44	19	7.1	15	15	4.3	5.2.

However, in that case hardly any reflexions with $k > 1$ appeared, and thus the data obtained from this crystal were too meager to be used in the structure determination.

A comparison of intensities for a large number of reflexions confirmed the correctness of the following parameter values (Table 1):

$$2\pi z_1 = 0; \quad z_1 = 0.$$

$$2\pi z_2 = 100^\circ; \quad z_2 = 0.278.$$

$$2\pi z_3 = -80^\circ; \quad z_3 = -0.222.$$

$$2\pi z_4 = -45^\circ; \quad z_4 = -0.125.$$

$$2\pi x_5 = 90^\circ \quad x_5 = 0.250 \quad 2\pi z_5 = 100^\circ; \quad z_5 = 0.278.$$

The accuracy of the parameter values is difficult to evaluate because of the predominant scattering of the lead atoms in most of the visible reflexions. However, the maximal error in z_2, z_3 and z_4 is probably 0.015 and in x_5 and $z_5 \sim 0.03$.

From powder photographs taken with Cu–K radiation ($\lambda_{K_{\alpha_1}} = 1.54050$ Å) in a 19 cm Bradley camera the following cell dimensions were calculated (Table 2):

$$a = 5.870 \text{ Å} \pm 0.003 \quad c = 5.494 \text{ Å} \pm 0.003.$$

Table 2

Powder photographs of Diaboleite, $Pb_2Cu(OH)_4Cl_2$. Unfiltered Cu–*K*-radiation but β-reflections omitted.

$I_{obs.}$	*h k l*	$\sin^2 \theta_{obs.}$	$\sin^2 \theta_{calc.}$	$I_{obs.}$	*h k l*	$\sin^2 \theta_{obs.}$	$\sin^2 \theta_{calc.}$
v.st.	0 0 1	0.0196	0.0197	m.	4 0 0	0.2757	0.2756
m.	1 1 0	0.0344	0.0345	m.	4 0 1	0.2956	0.2954
w.	1 0 1	0.0369	0.0369	st.	0 0 4	0.3149	0.3149
v.st.	1 1 1	0.0541	0.0541	m.	3 3 1	0.3301	0.3298
st.	2 0 0	0.0690	0.0689	m.	4 2 0	0.3433	0.3446
w.	0 0 2	0.0788	0.0787	st.	1 1 4	0.3490	0.3493
st.	2 0 1	0.0887	0.0886	m.	4 2 1	0.3640	0.3643
w.	1 0 2	0.0960	0.0959	m.	2 0 4	0.3829	0.3837
v.st.	1 1 2	0.1130	0.1131	m.	3 3 2	0.3885	0.3888
st.	2 2 0	0.1380	0.1378	m.	4 2 2	0.4242	0.4233
m.	2 0 2	0.1471	0.1476	v.v.w.	1 5 0	0.4474	0.4480
w.	2 1 2	0.1651	0.1649	m.	2 2 4	0.4523	0.4527
m.	3 1 0	0.1726	0.1723		4 0 3		0.4528
m.	0 0 3	0.1769	0.1771	m.	1 5 1	0.4677	0.4677
v.st.	1 3 1	0.1921	0.1920	m.	1 3 4	0.4875	0.4872
m.	1 1 3	0.2114	0.2116	m.	4 2 3	0.5214	0.5217
w.	2 2 2	0.2161	0.2165	m^+	1 1 5	0.5270	0.5265
st.	2 0 3	0.2458	0.2460		1 5 2		0.5267
st.	1 3 2	0.2512	0.2510				

Discussion of the structure

The interatomic distances are:

$$\begin{array}{llll} Pb - 4\,O & = 2.57\ \text{Å} \pm 0.1\ \text{Å} & O - 4\,O & = 2.94\ \text{Å} \\ Pb - 2\,Cl_2 & = 3.01\ \text{Å} \pm 0.05\ \text{Å} & O - 2\,Cl_1 & = 3.44\ \text{Å} \\ Pb - 2\,Cl_1 & = 3.18\ \text{Å} \pm 0.05\ \text{Å} & O - Cl_2 & = 3.03\ \text{Å} \\ Cu - 4\,O & = 2.07\ \text{Å} \pm 0.2\ \text{Å} & O - Cl_2 & = 3.88\ \text{Å} \\ Cu - 2\,Cl_1 & = 2.75\ \text{Å} \pm 0.05\ \text{Å} & Cl_1 - Cl_2 & = 4.18\ \text{Å} \end{array}$$

The lead atom is in contact with four oxygen atoms forming a square and with four chlorine atoms on the opposite side of the lead atom. The chlorine atoms are situated a little above and below the corners of another square parallel with the oxygen square and rotated 45° compared with it. Thus the configuration around the lead atom consists of a distorted, square Archimedean antiprism. The difference between the Pb–O and Pb–Cl distance is compatible with the difference in size between the O^{2-}-ion and the Cl^--ion (ionic radii are 1.4 and 1.8 Å respectively). In the cubic modification of PbF_2 the bond length for the coordination number 8 is 2.57 Å and the bonds are probably mainly ionic (2). As will be seen the length of the eight lead bonds in diaboleite agrees well with this distance if the difference in size between the F^--ion ($r = 1.33$ Å) and the Cl-ion and the O^{2-}-ion is taken into account, thus indicating that the lead bonds in diaboleite are also mainly ionic (A. BYSTRÖM, Dissertation, Stockholm, 1950).

The copper atom has a distorted octahedral coordination group with four O atoms at 2.07 Å and two Cl atoms at 2.75 Å. These distances differ only insignificantly from those found by WELLS (8) for one half of the Cu atoms in atacamite, $Cu_2Cl(OH)_3$, the four Cu–OH distances being in that case 2.01 Å and the two Cu–Cl distances 2.76 Å. As has been pointed out by Wells, the difference in length between the Cu–OH distances and the Cu–Cl distances is larger than would be expected from the differences in size of the atoms and must be due to some difference in the bonds.

The O–Cl distances are very short and might imply O–H–Cl bonds. It is, however, known that the Cl atom does not form H bonds as readily as the O atom and thus the H atoms may be situated between the 0 atoms in spite of the comparatively longer O–O distances. A comparison of the charges of the anions for the two alternatives gives the following result:

1. O–H–Cl bonds.	2. O–H–O bonds.
$O \;= \frac{4}{8} + \frac{2}{6} + \frac{1}{2} = 1\frac{1}{3}.$	$O \;= \frac{4}{8} + \frac{2}{6} + \frac{2}{2} = 1\frac{5}{6}.$
$Cl_1 = \frac{4}{6} + \frac{8}{8} = 1\frac{2}{3}.$	$Cl_1 = \frac{4}{6} + \frac{8}{8} = 1\frac{2}{3}.$
$Cl_2 = \frac{8}{8} + \frac{4}{2} = 3.$	$Cl_2 = \frac{8}{8} = 1.$

Obviously the assumption of O–H–O bonds gives a more satisfying distribution of the charges. However, even in that case the charges on the Cl_1 atoms are too high. By these calculations it has been assumed that the six Cu bonds are of equal strength but the long Cu–Cl_1 distances probably imply that these bonds are weaker than the Cu–O bonds. In fact, an ideal distribution of the charges would result if it is assumed that there are no bonds at all between the Cu and the Cl_1 atoms. In that case the structure is built up of layers of the composition $Pb_2Cu(OH)_4Cl_2$, and held together in the *c*-direction only by weak intramolecular forces. This agrees well with the perfect and brilliant cleavage parallel to {0 0 1}, but PALACHE's observation that this cleavage is not easily obtained indicates somewhat stronger forces between the layers. Hence it is probable that Cu–Cl_1 bonds exist but that they are considerably weaker that the Cu–O bonds.

This investigation was made in the course of a study on the structures of metal oxides, which is financially supported by the Swedish Natural Science Research Council.

SUMMARY

The crystal structure of diaboleite, $Pb_2Cu(OH)_4Cl_2$, has been determined. The cell is tetragonal with $a = 5.870$ Å (± 0.003) and $c = 5.494$ Å (± 0.003). The space group is C^1_{4v}–$P\,4\,mm$ and the atomic positions are:

2 Pb in 2 (*c*):	$\frac{1}{2}\,0\,z_1;\quad 0\,\frac{1}{2}\,z_1$	$z_1 = 0$
1 Cu in 1 (*a*):	$0\,0\,z_2$	$z_2 = 0.278$
1 Cl in 1 (*a*):	$0\,0\,z_3$	$z_3 = -0.222$
1 Cl in 1 (*b*):	$\frac{1}{2}\,\frac{1}{2}\,z_4$	$z_4 = -0.125$
4 O in 4 (*d*):	$x_5\,x_5\,z_5;\quad x_5\,\bar{x}_5\,z_5$	$x_5 = 0.250$
	$\bar{x}_5\,x_5\,z_5;\quad \bar{x}_5\,\bar{x}_5\,z_5$	$z_5 = 0.278.$

The hydrogen atoms probably form O–H–O bonds. A consideration of the interatomic distances shows that the Pb–O and Pb–Cl distances are compatible with ionic bonds. The oxygen atoms and chlorine atoms form distorted square Archimedean antiprisms around the lead atoms and distorted octahedra around the copper atoms.

University of Stockholm. Institute of Inorganic and Physical Chemistry, February 1950.

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The effect of egg extract on the surface of sea urchin eggs and spermatozoa

By John Runnström and Alberto Monroy

With 3 figures in the text

Extracts from frozen and thawed *Arbacia punctulata* eggs contain some agent which precipitates the jelly coat in intact eggs (9, 13). The present writers have repeated these experiments with *Arbacia lixula* from the Gulf of Naples. Eggs forming a dense suspension were frozen by aid of carbon dioxide snow. They were kept for several hours at —20° and then thawed at room temperature. The coarser residue was then centrifuged down and the supernatant diluted with equal parts of sea water. In eggs of Arbacia added to the mixture a rather refractive outline of the normally invisible jelly coat appeared.

Later frozen and thawed as well as frozen and dried eggs of *Arbacia lixula* were extracted with a 1-N KCl-solution. The KCl-extracts were then dialyzed against sea water. The extracts were tested with respect to their action on intact eggs of *Arbacia lixula* and *Paracentrotus* as well as on jelly solutions from these species, see Table 1. The freezing drying was carried out in the apparatus of Flossdorf and Mudd (1). The jelly solution was obtained in the usual way by brief treatment of the eggs with acidified sea water which was afterwards dialyzed against normal sea water. The dry weight of the jelly solution was not determined (~ 0.5 mg/ml). In the present case, the frozen and thawed Arbacia extract held a higher N-content

Table 1

Extracts in 1 N KCl were prepared from frozen and thawed and frozen and dried eggs of *Arbacia lixula* (AEX) and then dialyzed against sea water. Effect tested on jelly coats of intact eggs and on jelly solutions from *Arbacia lixula* and *Paracentrotus lividus* respectively.

	Effect on jelly coats of intact egg		Effect on jelly solution (0.1 ml AEX added to 0.5 ml j. s.)	
	Arbacia	Paracentrotus	Arbacia	Paracentrotus
AEX frozen and thawed 0.92 mg N/ml	Precipitation	No effect	Precipitation	Slight turbidity
AEX frozen and dried 0.64 mg N/ml	No effect		Turbidity	Turbidity

than the frozen and dried extract. This, however, is not the reason for the difference between the two as follows from observations on the effect of different dilutions of the extracts.

Extracts from frozen and thawed or frozen and dried Paracentrotus eggs had no precipitating effect on jelly solution and only a very weak effect on the jelly of intact Paracentrotus eggs. Conversely, Paracentrotus egg extract gave a strong precipitation and strong coagulation of Arbacia jelly coat, see Table 2. It must be pointed out, however, that the capacity of Paracentrotus egg extract to coagulate Arbacia jelly coat varied according to the material.

Table 2

Extracts in 1 N KCl were prepared from frozen and dried eggs of *Paracentrotus lividus* (PEX) and dialysed against sea water. Effect tested on jelly dissolved in sea water diluted to 50 per cent with dist. water and on jelly coats of intact eggs (in sea water). Crosses indicate different degrees of precipi tation of the jelly coat in intact eggs.

	Effect on jelly solution (0.1 ml PEX added to 0.6 ml j. s.)		Effect on intact eggs	
	Arbacia	Paracentrotus	Arbacia	Paracentrotus
PEX 2.4 mg/ml	Strong precipitation	Slight turbidity		
Dilutions with sea water				
2 : 1			+ + + +	+
1 : 1			+ + +	−
1 : 2			+ + +	−
1 : 4			+ +	−
1 : 9			+ +	−

No precipitation of the jelly was observed when extracts from eggs of *Psammechinus miliaris* were tested on eggs of the same species.

Most workers with egg material of sea urchins probably observed incidentally that the jelly coat may coagulate upon cytolysis of the egg. Wishing to study this phenomenon more closely, the present writers induced cytolysis by adding 12–15 ml distilled water to 2 ml suspension of unfertilized *Arbacia lixula* eggs. These were allowed to sink and thereafter the medium was brought back to the original volume by means of a pipette connected with a water pump. After 3—4 minutes the eggs were seen to burst at one point from which a slight outflow of cytoplasm occurred. Above this point the jelly coagulated, often presenting a number of different precipitated layers.[1] The coagulation extended only rarely beyond the region adjacent to the

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[1] Heparin inhibits the local coagulation of the jelly coat. Arbacia eggs were pretreated with 0.5 per cent heparin (Na-salt 80 int. units per mg). After 120 minutes the medium was diluted by distilled water containing 0.1 per cent heparin. In a parallel control without heparin the outflow of the cytoplasm was inhibited by local clotting of the jelly such as is represented in Fig. 1. In the presence of heparin no clotting of the jelly coat occurred. A stream of granules was seen to leave the exovate. In the beginning the granules seemed to be kept together by some invisible matrix, but soon they were dispersed in the medium as indicated by a lively Brownian movement. In the control the coagulation of the jelly coat extended gradually around the egg. In the presence of heparin no coagulation appeared even after prolonged exposure to the diluted medium.

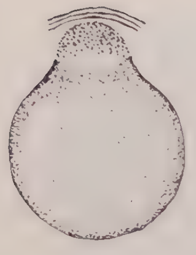

Fig. 1. Egg of *Arbacia lixula* broken up at one point upon exposure to strongly diluted sea water. Local coagulation of the jelly coat in contact with outflowing cytoplasm.

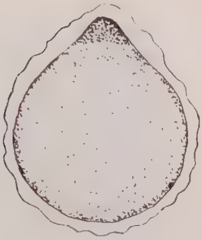

Fig. 2. Egg of *Arbacia lixula* broken up in strongly diluted sea water after pretreatment with egg extract. Coagulation of jelly coat all around the egg.

expressed cytoplasmic material, see fig. 1. The jelly coagulation is apparently caused by some agent diffusing from the interior of the cytoplasm. Upon addition of Arbacia egg extract to the diluted medium a rather strong coagulation of the whole jelly coat occurred. In one experiment for example, the eggs were transferred to diluted medium in the way described. The local coagulation occurred in the vicinity of cytoplasmic outflow. To 2 ml of egg suspension in diluted medium, 0.5 ml Arbacia egg extract containing 2.8 mg N/ml, was added. Now a general coagulation of the jelly coats was observed. Furthermore, owing to the stickiness of the jellies a strong agglutination occured.

In other cases the eggs were brought into diluted medium after previous treatment with Arbacia egg extract in sea water (e.g. 1.5 ml sea water + 0.5 ml extract). Upon dilution a coagulation of the jelly coat occurred all around the egg, see fig. 2.

In the case represented in Fig. 2 the surface of the jelly was irregularly folded but the underlying jelly was homogeneous. Sometimes the jelly coat showed a fibrillar coagulation as shown in Fig. 3.

The egg extracts used in connection with the dilution experiments were prepared by extracting the frozen and dried eggs with distilled water. The extract was then dialyzed against sea water.

Crystalline lysozyme (Armour & Co.) 0.05—0.1 per cent was added to sea water. No effect on the jelly coat was noted, but following dilution carried out as described above a strong coagulation of the jelly occurred. A similar effect was observed if lysozyme was added to eggs previously brought into diluted medium. The coagulation of the jelly in eggs subjected to egg extract and thereafter brought to diluted medium was reinforced upon addition of lysozyme.

The fibrillar coagulation shown in fig. 3 often occurred after pretreatment with lysozyme or after combined action of this substance and egg extract.

Pretreatment of the eggs in Ca-free sea water with lysozyme gave a still stronger coagulation and contraction of the jelly coat after dilution than pretreatment with lysozyme in Ca containing medium.

The point of rupture on the surface of the heparinized eggs appeared as a broad orifice through which great quantities of material had escaped.

Eggs of *Psammechinus miliaris* are much more disintegrated upon exposure to diluted medium than are Arbacia eggs. The presence of heparin favours this disintegration. After 100—120 minutes the heparinized eggs were dissolved in blisters and granules. The control eggs were strongly vacuolated and swollen but the cytoplasm was much less disintegrated.

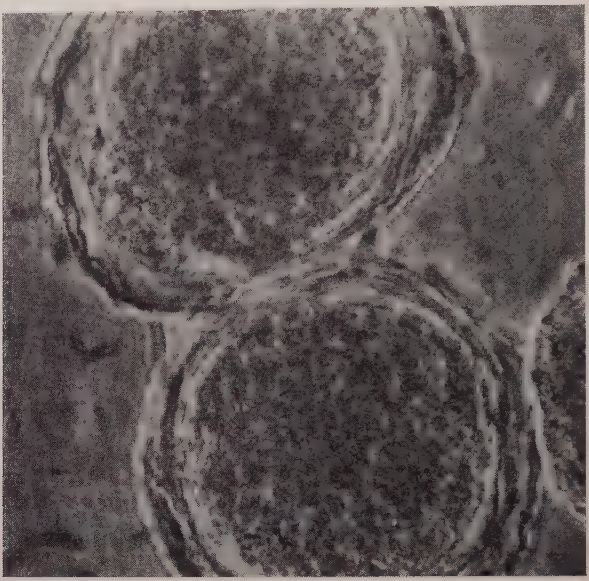

Fig. 3. Egg of *Arbacia lixula* subjected to the same treatment as in the egg represented in Fig. 2. Strong fibrous coagulation of the jelly coat. Phase contrast.

Conversely combination of lysozyme and extract from frozen and dried eggs had no precipitating effect on the jelly coat of intact eggs.

The coagulation of the jelly coat in Arbacia eggs occurring in diluted medium after pretreatment with Arbacia egg extract is stronger than that caused by addition of Paracentrotus egg extract to intact Arbacia eggs in sea water. The precipitation following exposure of Arbacia eggs to Paracentrotus egg extract in sea water is reinforced by dilution of the medium with distilled water.

In some experiments transfer of the lysozyme treated eggs to an isotonic sucrose solution proved to result in a coagulation of the jelly coat identical with that occurring in diluted medium. In the sugar solution the eggs did not break up.

On a visit to the Zoological Station in Naples in the spring 1949 Dr. T. Hultin kindly carried out a number of experiments on the jelly coagulation under the action of egg extracts and lysozyme. Extracts of frozen and thawed eggs in sea water as well as extracts in isotonic NaCl-solution of homogenate prepared in Ca free or Ca containing sea water gave a coagulation of the jelly coat of Arbacia eggs in isotonic salt medium. This was indicated by the appearance of a contour line on the surface of the normally invisible jelly coat. The coagulation became much stronger when the eggs were transferred into isotonic sucrose solution or distilled water. The Arbacia jelly coat reacted both to extracts from Arbacia and Paracentrotus eggs. Paracentrotus-eggs, on the other hand reacted only in medium with decreased ion concentration, diluted sea water or sucrose solution. When extracts from frozen and dried eggs were utilized a very weak or no contour line appeared on the surface of the jelly coats. On dilution, however, the coagulation occurred in the same way as described above. On the basis of his observations Hultin suggested that the ion concentration in the medium plays a great role in the coagulation of the jelly coat. Observations carried out in the meantime by Mr. Immers pointed in the same direction.

He found that a solution of jelly coat substance did not coagulate in the presence of lysozyme in sea water. On dilution, however, a strong coagulation occurred. The action of pure solution of NaCl, KCl, $CaCl_2$ and $MgCl_2$ did not differ from that of sea water. The cations seemed to have almost equal effects on the basis of molar concentrations. At 0.05 M the coagulation was already as strong as in distilled water.

Eggs of Echinus esculentus were subjected to the action of 0.05 per cent lysozyme in sea water. A strong contour line appeared on the surface of the jelly coat if the eggs were transferred to sucrose solution (devoid of lysozyme). If, however, the eggs were washed repeatedly with sea water before the transfer to the sugar no coagulation occurred.

As earlier reported (12) eggs of *Arbacia lixula* sometimes present a membrane which does not coagulate. This membrane does not elevate from the egg surface but remains attached to it as a thin gelatinous rim. This condition often arose in eggs which had been kept in sea water for some hours. It was demonstrated that crystalline serum albumin (Armour & Co) and also ATP restores the capacity. Egg extract of Arbacia had the same capacity, see table 3. The extract was active even when diluted 1: 20 (1.9 ml sea water + 0.1 ml egg extract).

Table 3

Water extracts were prepared from frozen and dried *Arbacia lixula* eggs (AEX) and dialyzed against sea water; 3.8 mg N per ml extract. Arbacia eggs were inseminated in the presence of extract and membrane formation watched. The jelly coats were removed from the eggs. One drop 0.1 per cent cryst. serum albumin to each sample.

	1 2 ml sea water	2 1.5 ml sea water 0.5 ml AEX	3 1.75 ml sea water 0.5 ml AEX
Only a gelatinous rim	87 per cent	—	2 per cent
Partial membrane formation	13 per cent	—	—
Membrane well elevated all around the egg	—	100 per cent	98 per cent
Number of eggs counted	54	54	42
Development after 60 hours	Swimming bilateral larvae	Development has stopped in an early blastula stage. Membrane still present in most of the eggs	

When the eggs remain in sea water containing egg extract the development stops in a late cleavage or beginning blastula stage. A coagulation of the hyaline layer seems the most probable explanation of the arrest of development. Possibly also the cytoplasmic surface is involved in the coagulation. Thus the surface movements necessary for cytoplasmic cleavage are inhibited. In pure sea water, on the other hand, the soft and elastic membrane did not prevent further development. It was gradually detached from the egg surface and dissolved in the normal stage of hatching. Even rather normal plutei may be raised from eggs with a nonelevated membrane.

Table 4

Water extracts were prepared from frozen and dried Arbacia (AEX) and Paracentrotus eggs (PEX) and dialyzed against sea water; AEX 3.8 and PEX 3.2 mg N per ml extract. Arbacia eggs were inseminated in presence of extract and fertilization and membrane formation watched. The jelly coats were removed from the eggs. One drop 0.1 per cent cryst. serum albumin to each sample.

	1	2	3
	2 ml sea water	1.5 ml sea water 0.5 ml AEX	1.5 ml sea water 0.5 ml PEX
No fertilization	—	—	82 per cent
Only a gelatinous rim	75 per cent	12 per cent	15 per cent
Asymmetric membrane	15 per cent	8 per cent	3 per cent
Membrane well elevated all around the egg	10 per cent	80 per cent	0 per cent
Number of eggs counted	71	74	91
Development after 16 hours	Swimming blastula	Development has stopped in early blastula stage	

As follows from Table 4, extracts from Paracentrotus eggs had not the ability of improving the membrane elevation upon fertilization, see also (12). In this experiment the jelly coat was removed and a small quantity of serum albumin added to protect the naked egg (11).

In a parallel experiment with Paracentrotus extract and Arbacia eggs the number of eggs with gelatinous rim approached 50 per cent. No membrane elevation and coagulation occurred; 4 ml distilled water were added to the 2 ml sample with Paracentrotus extract. The eggs swelled but the cytoplasmic surface remained intact. The membrane was detached and attained the appearance of an almost normal fertilization membrane.

The experiment recorded in Table 5 shows that the fertilization of Paracentrotus eggs is inhibited both by Paracentrotus and Arbacia extracts. This inhibition proved, however, to be reversible when the eggs were transferred to pure sea water.

In one experiment extracts from unfertilized Paracentrotus eggs and from late cleavage stages (7 hours after fertilization) were compared; 1.8 gr of the frozen and dried material were extracted in both cases with 100 ml distilled water. Thereafter the extracts were dialyzed against sea water. The final solution was diluted 1: 2 or 1: 4 in the experiments. Only a very low percentage of fertilization or none was observed upon insemination of Paracentrotus eggs in the presence of extract from unfertilized eggs. In the few fertilized eggs only nuclear but no cytoplasmic cleavage occurred. It was noted that the spermatozoa only with difficulty penetrated the jelly. From this observation it may be inferred that the egg extract causes a certain modification of the colloidal state of the jelly coat even if no visible coagulation is brought about. It was often stated that the extract from unfertilized eggs inhibits the swelling or gradual dissolution of the jelly coat which occurs in eggs left in pure sea water.

Table 5

Water extracts were prepared from frozen and dried Arbacia (AEX) and Paracentrotus (PEX) eggs and dialyzed against sea water; AEX 3.8 and PEX 3.2 mg N per ml. Paracentrotus eggs were inseminated in presence of extract and fertilization and membrane formation watched. The jelly coats were not removed. No jelly coagulation occurred.

	1	2	3
	2 ml sea water	1.5 ml sea water 0.5 ml AEX	1.5 ml sea water 0.5 ml PEX
Observations on the inseminated eggs	100 per cent normal membrane formation	No fertilization even after repeated insemination	5–10 per cent membrane formation, strong contraction of the fertilized egg. The remainder unfertilized
About 15 minutes after insemination eggs (in 2 and 3) transferred to normal sea water and inseminated		Almost 100 per cent normal fertilization	

The effect of extract from the 7 hour stage of development differed in certain respects from what has been recorded in the preceding description. The number of fertilized eggs was about 15 per cent. The jelly coat swelled in the course of 3 hours and finally disappeared. Despite the more liquid state of the jelly coat at the moment of insemination the percentage of fertilized eggs was comparatively low. This shows that the inhibitory effect is exerted rather on the surface of the eggs or of the spermatozoa than on the jelly. After prolonged pretreatment of the eggs in the extract from the 7 hour stage the vitelline membrane seems to dissolve. At fertilization no membrane appeared but cortical granules were expelled. Both the modification of jelly coat and the dissolution of the vitelline membrane are probably due to the presence of a proteolytic enzyme in the extract from the 7 hour stage. It is known (3, 4) that a proteolytic enzyme is active in the blastula stage. This enzyme is instrumental in the removal of the fertilization membrane occurring at hatching.

Still another effect of egg extracts has been recorded in table 6. In combination with "egg water" which contains jelly substance in solution the egg extract brings about a strong tail agglutination. The radially arranged clusters arise in the following way. Initially groups of spermatozoa are agglutinated by their heads. When the spermatozoa are going to separate they stick together in the presence of egg extract by the ends of their tails. Thus small clusters of spermatozoa arise with their heads directed towards the centre. Additional spermatozoa are, however, incorporated into the clusters. The radial arrangement remains but the later incorporated individuals often have their heads directed outwards.

Not only frozen and dried but also frozen and thawed Arbacia egg extracts give the effect recorded in Table 6.

Paracentrotus egg extract (3.2 mg N/ml) 1 part + 4 parts P. egg water gave a tail agglutination of Paracentrotus spermatozoa.

Experiments with a Paracentrotus extract (2.3 mg N/ml) showed that this had

Table 6

Agglutination of spermatozoa of *Arbacia lixula* in "egg water" or in "egg water" combined with *Arbacia lixula* egg extract (AEX) in sea water, 3.8 mg N per ml. The egg water was removed from an egg suspension in sea water. The reaction was studied according to F. R. LILLIE's (5) method. One drop of sperm suspension (1 drop dry sperm to 5 ml sea water) and one drop egg water or egg water + egg extract were brought to mix between slide and coverslip.

1 Egg water	2 5 parts egg water 1 part AEX	3 5 parts sea water 1 part AEX
Immediate head agglutination lasting for 20 seconds. Thereafter some small groups of spermatozoa agglutinated by the tails. This tail agglutination disappeared gradually	Immediate head agglutination lasting for about 30 seconds succeeded by tail agglutination which increases in the course of the following 5 minutes. Characteristic clusters with radially arranged spermatozoa	Even after 10 minutes no, or very weak, tail agglutination

no activity beyond a 1: 10 dilution, whereas the limit in the case of an Arbacia extract with the same N-content was found to be the dilution 1: 1000.

Cross tests (Paracentrotus sperm + Arbacia egg extract and vice versa) carried out at different seasons have not been consistent. Further experiments must be awaited.

Different attempts were made towards purification of the active agent present in egg extracts but so far these have not been successful. The writers first assumed that the precipitating agent of the egg extract might be a basic protein like the jelly coagulating agent isolated from spermatozoa by HULTIN (2). The same procedure was therefore adopted as in HULTIN's work.

The water extract was centrifuged and dialyzed. It was brought to pH 10. A flocculent precipitate was formed in Paracentrotus, but not in Arbacia extracts. After removal of precipitate if any and dialysis the extract was concentrated in vacuum.

Upon addition of a concentrated solution of picric acid a precipitate was formed which was partly dissolved in 80 per cent acetone. The solution was precipitated with 20 per cent H_4SO_2. After centrifuging and washing with alcohol and alcohol + ether, the precipitate was dissolved in slightly alkaline distilled water and finally dialyzed against sea water.

The above procedure was hardly appropriate as the egg extracts proved to be no simple basic protein when opportunity later was given to submit the extracts to electrophoresis. The picric acid precipitation did not seem to increase the jelly coat coagulating activity of the extract per mg N. The activity seemed rather to be somewhat decreased — an accurate assay has not been carried out. Anyhow it is of interest that the activity was not altogether annihilated by the rough treatment involved in the procedure described above. The active agent can thus hardly be a more complicated protein. Histamine on the other hand gave no effect.

Water extracts of unfertilized Arbacia eggs were fractionated by adding $(NH_4)_2SO_4$ to 30, 50 and 100 per cent saturation. So far no difference could be demonstrated when comparing the fractions with repsect to their capacity of causing tail agglutination of Arbacia spermatozoa in the presence of Arbacia egg water. This preliminary report of work which has to be extended, points to the conclusion that the agent active in tail agglutination forms no precipitable fraction but is probably carried down by adsorption of the precipitates.

The enzyme plasmin combined with egg water proved also to bring about a tail agglutination of the spermatozoa. Plasmin was kindly prepared for us by Dr. G. LUNDBLAD. The method described by LOOMIS et al (7) was used. A one per cent solution of the preparation had an activity of 29.10^{-8} units measured according to Hultin, see Lundblad (6). Two parts egg water were mixed with one part 0.3 per cent plasmin in sea water. The final concentration of the enzyme was thus 0.1 per cent ($2.9.10^{-8}$ units). This brings about a tail agglutination of the same type as described above, if egg water or eggs with jelly coats are present.

The tail agglutination was observed incidentally in numerous experiments in which eggs of Arbacia, Paracentrotus or Psammechinus were fertilized in 0.075–0.15 per cent solutions of plasmin in sea water. The egg water was in this case provided by the jelly coats covering the eggs.

Serum albumin was never observed to cause tail agglutination, not even in conjunction with egg water.

Discussion

Notwithstanding the preliminary nature of this report certain general points of view emerge from the facts presented.

The interior of the sea urchin eggs evidently contains agents which are able to cause coagulation of the jelly coat. The most direct demonstration of this was given when extracts in sea water from frozen and thawed Arbacia eggs were allowed to act on the jelly coat of the same species. This result has not been reproduced in other sea urchin species so far examined. However, in all of them a tendency towards coagulation of the jelly coat was predominant upon cytolysis of the egg. Thus cytolysis releases coagulating agents present inside the cytoplasm in these species.

Addition of extracts from frozen and dried eggs does not cause jelly coagulation of intact eggs in sea water. Freezing and drying preserves the chemical and colloidal state of the protoplasm much better than freezing and thawing. This latter procedure involves considerable chemical and structural changes which like cytolysis may release or activate agents causing jelly coagulation. On the other hand, the extracts from frozen and dried eggs have a pronounced effect on jelly coats of eggs which cytolyze in a hypotonic medium or are transferred into sucrose solution.

The reaction: jelly + frozen-dried extract (I) is more sensitive to ions than the reaction: jelly + frozen-thawed extract (II). An increase of the salinity of the sea water beyond the normal, however, impaired or inhibited the reaction II (observation due to Dr Hultin). The reaction II proceeded in the case of Arbacia eggs both on addition of Arbacia or Paracentrotus extract. When Paracentrotus eggs were used both reaction I and II were inhibited in sea water, but they occurred after the transfer of the egg into a medium of lower electrolyte content. Even when no coagulation occurs a certain reaction between the two components must be assumed, but the linkages formed are rather weak.

The coagulation of jelly discussed here is no enzyme reaction. Lysozyme reacts with the strongly acid jelly, see for references (10), by virtue of its basic character. Likewise the agent of the egg extract probably has certain reactive basic group although the substance as a whole did not show a basic character. It would be premature to discuss the species specificity of the agents contained in the extracts on the basis of the present evidence. The differences observed seem rather to reside in the different character of the jelly coats in the two species concerned. The Arbacia jelly coat may possibly have a more acid character than that of Paracentrotus.

Lysozyme does not promote the membrane formation upon fertilization as does the water extract of eggs. Conversely the membrane remains low and granular in eggs fertilized in the presence of lysozyme, see (10 p. 278). Unlike egg extracts lysozyme does not affect the surface of the cleaving eggs. It allows a normal development until advanced stages. Not even in conjunction with egg water did it bring about a tail agglutination of the spermatozoa. Under the conditions specified above, lysozyme proved to exert the coagulating effect in all the species so far examined. Thus the action of lysozyme seems to differ in many respects from that of the agent of the egg extract although their jelly coagulating effect is similar.

Sperm extracts obtained by heating or acidifying sperm suspensions in sea water coagulate the jelly coat, for references see (10, p. 264). According to HULTIN (2) the active agent in the sperm extract is a basic protein. Its effect can be imitated by protamines, clupein or salmine. The coagulating effect of these strongly basic proteins is not affected at the ionic concentration of sea water.

MIRSKY (8) observed that the solubility of the proteins in 1 N KCl solution was lower in frozen and thawed than in frozen and dried unfertilized eggs of *Arbacia punctulata*. The former were comparable with respect to the solubility of their proteins with frozen and dried fertilized eggs. Mirsky's observations indicate that upon freezing and thawing some agent is released which favours the coagulation of the cytoplasm. This on the other hand points to the conclusion that the coagulation of the jelly coat described above reflects interior changes of the egg cytoplasm.[1]

Coagulation plays an important role also in the formation of the fertilization membrane. A number of different observations point to the conclusion (10, p. 278 and 12) that this coagulation is due to the release of certain agents from the interior of the egg. As also reported above an initiated membrane formation may be completed upon immersing the egg (Arbacia) in diluted medium. This may be accounted for by the release of certain agents from the interior of the egg. The coagulation was sometimes limited to smaller regions of the membrane. Under these conditions the parts of the jelly coat lying outside coagulated membrane regions were stratified in the way shown in Fig. 1. Apparently local streams of coagulating agents had affected first the membrane and then passing this the adjacent parts of the jelly coat as well. From previous results (12) it follows that the release of membrane coagulating substance in intact eggs is connected with a certain contraction of the egg surface.

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[1] In a series of experiments eggs deprived of their jelly coats were subjected to hypotonic cytolysis after previous exposure for example to lysozyme. The appearance of these eggs was markedly different from that of the control eggs. The cytoplasm was strongly vacuolated but at the same time more coagulated than in the control. There seems to be a connection between the vacuole formation and the coagulation as follows also from data presented in a paper in press (RUNNSTRÖM and KRISZAT, Exp. Cell Res., 1, 285, 1950). Thus lysozyme does not only favor the coagulation of the jelly coat but also that of cytoplasmic components, probably mainly fibrous proteins. The vacuoles, on the other hand, arise by swelling of granular components rich in lipids.

The agents in egg extract removing the inhibition of the membrane formation may do so by reacting with the inhibitory substance. This recalls the reaction between the jelly solution and a component in the egg extract. This reaction may block the inhibitory groups of the jelly or of the mucopolysaccharide residing in the egg surface, see also (10, 12). A similar reaction with the egg surface, primarily the hyaline layer, stops the development in an advanced cleavage or blastula stage. This may again be accounted for by a combination with mucopolysaccharides present, according to Monné, in the hyaline layer, see (10). The reaction of the egg extract with the egg surface or the hyaline layer is not impaired at the ion concentration of the sea water.

The tail agglutination described above is caused by a joint action of dissolved jelly substance and egg extract. It is also indicative of some reaction occurring between jelly and an agent present in the extract. The tail agglutination involves that the ends of the tails become linked to each other, i.e. the mechanism is the same as that postulated to occur in coagulation processes (10). Possibly the tail agglutination may be regarded as a model of how the reaction between an agent present in the egg extract and a mucopolysaccharide, the jelly substance, is able to cause a coagulation of cell components. From this point of view the phenomenon may prove to be of considerable interest.

Addition of lysozyme to Arbacia egg water caused no tail agglutination of Arbacia spermatozoa. Neither did it affect the titer of the sperm agglutinating action of egg water. This is an additional proof that lysozyme and the egg extract agent combine with the jelly substance in different ways.

The above considerations favour the contention that certain agents present inside the cytoplasm are able to induce coagulation of cytoplasmic components, of the vitelline membrane and of the jelly coat as well. In the unfertilized egg at least one of the components must be present in a bound or inactivated state. The release of this agent initiates the coagulation process. The same mechanism as that revealed under rather crude experimental conditions may participate in bringing about the colloidal changes occurring subsequent to fertilization or artificial activation.

SUMMARY

Extracts from frozen and thawed eggs of *Arbacia lixula* in sea water cause a coagulation of the jelly coat surrounding the eggs of this species. Extracts from frozen and dried eggs have no such effect on the jelly coat of Arbacia or Paracentrotus eggs.

Extracts from *Paracentrotus lividus* precipitate to a varying extent the jelly coats or jelly solution from Arbacia, but not from Paracentrotus eggs.

The coagulation of the jelly coat occurring upon hypotonic cytolysis of the egg has been studied. In general this coagulation is restricted to the region of the jelly coat which comes in direct contact with outflowing cytoplasmic material. Heparin inhibits this coagulation (foot-note p. 406). Conversely the whole jelly coat of cytolyzing Arbacia eggs coagulates after pretreatment with Arbacia egg extract. This occurs also if the extract is added after cytolysis of the eggs.

Lysozyme has no apparent effect on the jelly coat of intact eggs but a strong jelly coagulation occurs when immersing the eggs in dilute medium after pretreatment with lysozyme. It also brings about coagulation if added to eggs already cytolyzed in hypotonic medium. The described lysozyme effect was found in all the

species examined (*Arbacia lixula, Paracentrotus lividus, Psammechinus miliaris* and *microtuberculatus*).

The coagulating effect of egg extract or lysozyme on the jelly is enhanced if the eggs are transferred into isotonic sugar solution. The coagulation of the jelly is favored by decreasing the electrolyte concentration of the medium.

It often occurred that the fertilization of Arbacia eggs entailed the formation of a gelatinous rim adhering to the egg surface instead of a normally elevated membrane. Addition of Arbacia egg extract prior to fertilization reestablished normal conditions. However, the extract stops the development in a late cleavage or early blastula stage.

In conjunction with egg water, egg extract causes a tail agglutination of spermatozoa both in Arbacia and Paracentrotus. The proteolytic enzyme plasmin proved to have a similar effect in presence of egg water or jelly substance.

The release of agents similar to those present in the egg extracts may participate in bringing about the changes in colloidal state occurring subsequent to activation of the egg.

From the Wenner-Gren Institute, University of Stockholm, Stockholm and Stazione Zoologica, Naples.

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Read 25 January 1950

On the amylase-substrate compounds

By Karl Myrbäck

With 8 figures in the text

The belief seems to be well founded that the sole action of the amylases on their natural substrates, starch and glycogen, is the rupture of α-glucosidic 1,4-linkages, i.e. the characteristic glucosidic linkage of these substances (the "maltose linkage") (1). The relative number of this type of glucosidic linkage is about 95 % in ordinary starch and about 90 % in most types of glycogen. 5 % of the linkages in starch and about 10 % in glycogen are of an other type, and it is now quite clear that these linkages are mainly if not exclusively α-glucosidic 1,6-linkages ("isomaltose linkages"). Not only are these "anomalous" linkages not attacked by the normal amylases, but they also constitute obstacles to the amylase action, making certain parts of the molecules in their vicinity inaccessible to the enzymes (2). "Normal" maltose linkages in the vicinity of the "anomalies," although in themselves of the very type on which the amylases specifically act, are rendered stable by the adjacent "anomalies". The reason must be that the "anomalies" more or less prevent the enzyme-substrate combination (steric hindrance). In the following an attempt is made to explain, on the basis of this general hypothesis advanced long ago by the author (3), the action of the various amylases.

At the present time there seems to be no serious disagreement concerning the action of the β-amylase on starch and glycogen. The non-ramified molecules of the amylose are 100 % saccharified, and in the case of the branched molecules of amylopectin and glycogen, the enzyme brings about the saccharification of the non-reducing end-chains. At the ramification points, i.e. the isomaltose linkages, the enzyme action ceases, and the branched part of the molecules are left as high molecular limit dextrins, "amylodextrin," or β-dextrin. The β-amylase evidently attaches itself to the terminal maltose unit of the chains and liberates maltose molecules successively. If a chain molecule contains maltose linkages exclusively, it is broken down completely to maltose, provided that the chain contains an even number of glucose units. The shortest chain split by the enzyme is one with four glucose units. If the chain molecule contains an uneven number of glucose units, it is broken down to maltose plus one molecule of the trisaccharide maltotriose, which is not hydrolyzed by the enzyme.

The enzyme attaches itself only to the non-reducing end-group of a chain molecule. The presence of a reducing, pseudoaldehydic group has no fundamental importance; it can be oxidized to a carboxyl group without impairing the enzyme action (4).

Fig. 1 A gives a schematic representation of the attachment between β-amylase and a chain molecule. It can well be said that the current ideas concerning the en-

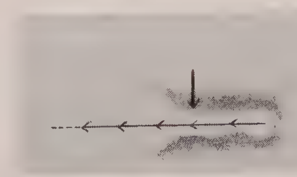

Fig. 1 A.

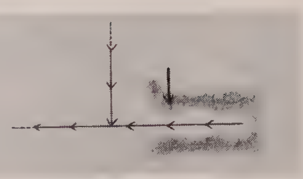

Fig. 1 B.

zyme-substrate combination were originally expressed by EMIL FISCHER in the simile of the lock and key. It is believed that the existence, in the enzyme molecule, of a group capable of combining with a group in the substrate is not a sufficient explanation of the occurrence of those special enzyme-substrate compounds in which the enzymic reaction takes place. On the contrary, it is necessary in most cases to assume several substrate-binding groups in the enzyme molecules acting on correspondingly situated groups in the substrates. In other words, we assume the existence, in the enzyme molecules, of "binding areas" with definite geometrical patterns of substrate-binding groups ("the web of the key"), on which the substrate molecules can be arranged in such a way that their electronic arrangement is so strongly influenced, that the enzymic reaction takes place. It is quite clear that even comparatively small changes in the structure of the substrate may interfere with its attachment to the enzyme, and the high substrate specificity is, as pointed out already by Fischer, easily understood on the basis of such assumptions.

In the case of the β-amylase, it appears safe to assume that an interaction takes place between substrate-binding groups of the enzyme and the terminal maltose unit of the substrate molecule. This interaction causes a weakening and finally a cleavage of the next to last glucosidic linkage of the chain. In the figures, the dark areas represent the substrate-binding groups on the enzyme surface. It is easily understood that from a branched molecule, as in Fig. 1 B, only one maltose molecule can be split off. The side chain will prevent any further action of the enzyme because the new end-group (after removal of the first maltose unit) can not be fitted into place on the binding area.

All investigations on the β-amylase action are in agreement with the hypothesis that amylopectin and glycogen have branched chains, but they do not allow us to decide between the different types of branched formulas which have been proposed (1, 5, 6, 7). K. H. MEYER (7) has advanced several reasons for a quite irregular formula, with multiple branching, which seems to explain very well all known facts about the polysaccharides in question. The present author has pointed out that at the moment no other formula can, without additional hypotheses, be brought into agreement with our knowledge concerning the enzymic synthesis of the polysaccharides (1, 8, 9).

The action of the α-amylases on starch and its components is more difficult to understand. Furthermore, not all α-amylases have the same action on the substrates (1). It may be that the final products are the same for all α-amylases, but the reaction mechanism is different. We can distinguish between (at least) two types of α-amylases: the malt α-amylase, which seems to be a special type, and the other α-amylases, which are more or less alike.

Curve 1 in Fig. 2 shows the yield of maltose from the action of β-amylase on starch. The limit of saccharification is about 60 %. Curve 2 shows the yield of reducing saccharides, calculated as maltose, with malt α-amylase, and Curve 3 is the correspond-

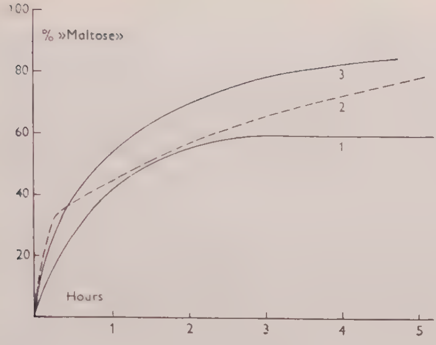

Fig. 2.

ing curve for the other α-amylases. It should be emphasized that the form of Curve 2 is not caused by the physical condition of the substrate, as for instance by retrogradation of the starch solution during the experiment. If the same starch solution is treated with malt α-amylase in one experiment, and with pancreatic amylase or takadiastase in a parallel experiment, we get the two different curves shown in Fig. 2. In this connection it should be mentioned that a curve identical to that found for crude malt α-amylase, was found by SCHWIMMER and BALLS (10) using crystalline malt α-amylase.

The action of the malt α-amylase clearly consists of two steps: an initial rapid stage, in which about 17 % of the glucosidic linkages of the substrate are ruptured, and a second, much slower reaction which proceeds for a long time and finally ceases when about 50 % of the linkages are hydrolyzed. In the first rapid reaction the viscosity of the starch solution decreases sharply and the color reaction with iodine vanishes. On the other hand, only small amounts of fermentable sugar are produced, perhaps 10 %. About 90 % of the starch is transformed into products, α-dextrins, showing chain-lengths of 5–10 glucose units. Thus the action of malt α-amylase begins with a rapid dextrinization of the polysaccharides. After that, a slow saccharification takes place, the enzyme obviously being capable of splitting the α-dextrins into smaller molecules, but at a considerably reduced velocity. The author has, for instance, compared the velocity of the hydrolysis of a high molecular substrate with that of a hexasaccharide with maltose linkages (4). The ratio of the velocities was 1 : 0.17.

It should be emphasized that the same type of glucosidic linkage, the α-1,4-linkage, is split in both phases of the malt α-amylase action. The great difference in the reaction rates of the two phases is not due to differences in the type of linkage but to the different position of the linkages in relation to the anomalies of the molecules.

If the mixture of α-dextrins produced from starch is separated by alcohol precipitation into several fractions, it is found that the fractions of lower degree of polymerization (5–7 units) are completely saccharified by β-amylase. These α-dextrins evidently contain maltose linkages only, and are called "normal α-dextrins." The fractions with somewhat higher molecular weight (the "anomalous α-dextrins," containing 8–10 glucose units) give, when treated with β-amylase, partly fermentable sugars and partly nonfermentable limit dextrins. These limit dextrins contain the isomaltose linkages of the substrate.

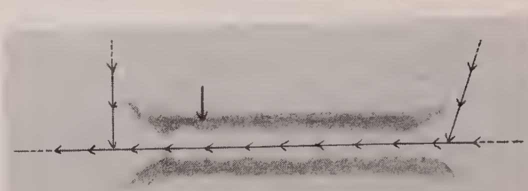

Fig. 3.

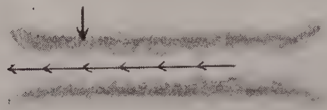

Fig. 4.

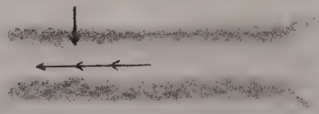

Fig. 5.

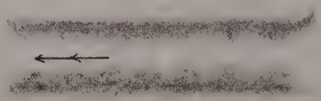

Fig. 6.

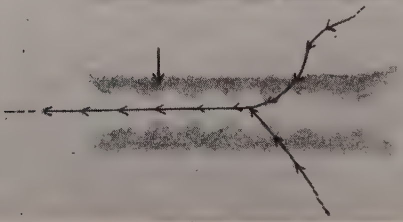

Fig. 7.

Obviously the malt α-amylase molecules are able to combine with interior parts of chain molecules far removed from end-groups: the enzyme is an endo-amylase. It splits only maltose linkages, the isomaltose linkages being recovered in the α-dextrins and, after the slow saccharification, in the limit dextrins.

The fact that a hexasaccharide, even if it contains maltose linkages exclusively, is split very slowly compared to a long chain substrate molecule is due to differences in the affinity of the enzyme for the substrates. MYRBÄCK and JOHANSSON (11) have compared the affinity constants, according to Michaelis and Menten, and found that the constant for a mixture of saccharides with 6 units on the average is less than 7 % of the affinity constant enzyme-starch. The form of the hydrolysis curve shows that the velocity in the case of starch is high until about 17 % of the linkages have been split, that is until the α-dextrins produced have an average chain length of about 8 units. It is concluded that the maximum affinity of the enzyme for certain parts of a substrate molecule is exerted if the enzyme has access to a section where at least 6–8 glucose residues are united with maltose linkages, and where no end-groups, branching points, substitution with phosphoric acid, or any other anomalous elements of constitution occur. It appears that the enzyme, in order to show its maximum activity, has to arrange about 6–7 glucose units of a

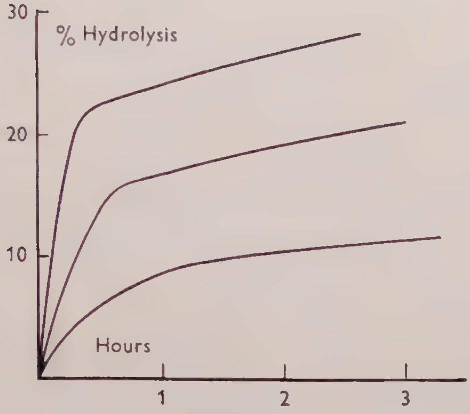

Fig. 8.

substrate molecule in a distinct pattern on its surface. This is shown schematically in Fig. 3. No one knows which linkage is hydrolyzed in a case such as this, but we may assume, for the sake of argument, that the position of the maximum weakening of a glucosidic bond is indicated by the arrow. End-groups, side-chains, or any other anomalies occurring in the part in question of the substrate molecule more or less disturb the arrangement of the substrate molecule on the binding area of the enzyme, decrease the affinity, and decrease the velocity of hydrolysis. However, even short chains are hydrolyzed at a low velocity. Therefore, we may assume that a hexasaccharide, for instance, can be fixed in the binding area as shown in Fig. 4. In this case, only part of the substrate-binding groups can act, and the affinity is decreased, but the fixing of the chain molecule on the enzyme is nevertheless strong enough to cause a sufficient weakening of a glucosidic bond to produce hydrolysis. Since the enzyme produces glucose as a primary product (even from a saccharide with only three units), we have to assume that an arrangement of such a molecule as shown in Fig. 5 will be sufficient. On the other hand, maltose is not attacked. The number of substrate-binding groups acting in this case (Fig. 6) must be too low to produce hydrolysis.

If in a branched molecule the free length between two ramification points is shorter than 8 units, the molecule probably can be arranged as shown in Fig. 7. However, the arrangement will be distorted in this case, and the resulting affinity and velocity of hydrolysis will be decreased. If the distance between the side-chains is very short no reaction at all will occur. Therefore, the number of linkages easily hydrolyzed by the enzyme will be dependent on the degree of branching of the substrate. This has been found to be the case. The three curves in Fig. 8 show that in amylose about 22 %, in starch about 17 %, and in glycogen and in β-dextrin from starch about 10 % of the linkages will be hydrolyzed with the maximum velocity. P. Bernfeld (12) has pointed out that, especially in the case of amylose, the retrogradation of the substrate may be of importance. However, to the best of the authors' knowledge, retrogradation has nothing to do with the above results. If retrogradation were the cause for only 22 % of the linkages of amylose being split at a high velocity, we should have, at this degree of hydrolysis, a substantial amount of high molecular substances left in the solution. On the contrary, practically all substance could be recovered as α-dextrins with an average chain-length of about 6 units (13).

In other amylases, as for instance in the animal amylases, the difference in affinity for long and short chain saccharides is less prominent. Correspondingly, the difference in velocity of the dextrinization and the saccharification is smaller and the hydrolysis curves show no definite breaks (Fig. 2).

As far as we know, the only action of an amylase is the hydrolysis of the maltose linkage. The isomaltose linkages are not attacked, and therefore give rise to the limit dextrins. Because of the mode of attachment of the enzyme to the substrate molecules, a few maltose linkages, in the vicinity of branching points and other anomalies, are more or less protected against the amylase action. The limit dextrins isolated after normal reaction times therefore contain mainly 3–6 glucose units. If the amylases are allowed to act for a very long time, more and more fermentable sugar is split off from these dextrins and isomaltose may be found as the ultimate product. The formation of this sugar after degradation of starch with takadiastase was demonstrated by AHLBORG and MYRBÄCK (14). The sugar was isolated in crystalline form by MONTGOMERY, WEAKLEY and HILBERT (15).

Institute of Organic Chemistry and Biochemistry, University of Stockholm.

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Vorgelegt am 7. Juni 1950.

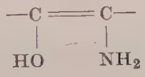
Aminoreduktone, Endiamine, Endithiole, Enole und Redukton-Derivate

Von Hans von Euler, Hans Hasselquist und Armin Glaser

Unter den oben genannten, zur Klasse der Reduktone gehörenden Stoffen sind mehrere, in früheren Mitteilungen noch nicht erwähnte, von erheblichem biochemischem Interesse. Im Folgenden soll ihr an Tillmans Reagenz geprüftes Verhalten beschrieben und mit den übrigen Eigenschaften, durch welche sie als Reduktone charakterisiert sind, verglichen werden.

I. Aminoreduktone

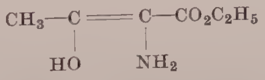
In einer vor kurzer Zeit erschienenen Übersicht[1] haben wir unter den Aminoreduktonen, welche die Gruppe



$$-\underset{\mathrm{HO}}{\mathrm{C}}=\underset{\mathrm{NH_2}}{\mathrm{C}}-$$

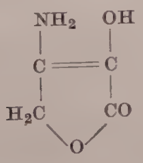
enthalten, folgende erwähnt:

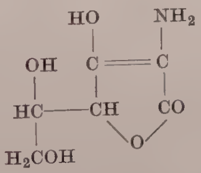
1. Der α-Amino-β-ketobuttersäure- ester bezw. dessen Enolform ist nach Martius und Knoop[2] einer der am stärksten reduzierenden organischen Stoffe überhaupt:



$$\mathrm{CH_3-}\underset{\mathrm{HO}}{\mathrm{C}}=\underset{\mathrm{NH_2}}{\mathrm{C}}\mathrm{-CO_2C_2H_5}$$

2. Amino-tetronsäure und 3. Scorbaminsäure





sind nach Micheel und Mittag[3] durch eine hohe Reduktionsfähigkeit ausgezeichnet: Amino-tetronsäure wie auch Scorbaminsäure[1] verbraucht 2 Atome Jod in saurer Lösung und reduziert saure Silbernitratlösung in der Kälte.

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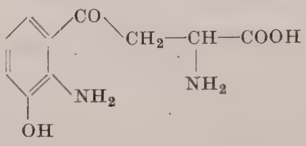
[1] Euler und H. Hasselquist, Reduktone, ihre chemischen Eigenschaften und biochemischen Wirkungen. Stuttgart, Enke, 1950.

[2] Martius und Knoop, Z. physiol. Chem. 240, 195 (1936).

[3] Micheel und Mittag, Z. physiol. Chem. 247, 34 (1937).

Über das Auftreten einer Enol-(Aminoredukton-)Form beim *Uramil* haben wir noch keine Erfahrung.

3-*Oxy-Kynurenin*, ein aromatisches Aminoredukton, ist in biologischer Hinsicht



besonders bemerkenswert; dieses lebenswichtige, im Tryptophanstoffwechsel auftretende Zwischenprodukt, das von BUTENANDT[2] als Gen-abhängig erkannt wurde, ist u. a. bei der biologischen Bildung der Nikotinsäure wirksam (Zs. f. Naturforsch. 4 b, 342 (1949)).

Die Reduktionsfähigkeit des 3-Oxy-Kynurenins scheint derjenigen der übrigen bis jetzt bekannten Amino-Reduktone ähnlich zu sein. Zunächst ist auffallend, dass dieser Stoff im Gegensatz zu allen bekannten Endiolen, Tillmans-Reagenz *nicht reduziert.*

Wir haben deswegen sein Verhalten zu Jod untersucht. 1,7 mg Sbst. wurden in 10 ml Wasser emulgiert und mit einer 0,008-norm. Jodlösung (Stärke als Indikator) titriert. Nach Verbrauch von 1,84 Mol. Jod per Mol. Sbst. verlief die Reaktion sehr langsam. Durch Zugabe von NaAc wurde die Reaktion beschleunigt (Entfernung von freiem HJ). Der Endwert von 3,7 Mol. Jod per Mol. Sbst. war sehr unscharf, da sich die Lösung stark braunrot färbt. Der grösste Teil des verbrauchten Jods dürfte in den Benzolkern eingetreten sein.

o-Amino-phenol

Obiges, an Oxy-Kynurenin erhaltenes Ergebnis hat uns veranlasst o-Aminophenol als Beispiel eines aromatischen Amino-reduktons auf sein Verhalten zu TR und zu Jod zu prüfen.

Das durch Umkrystallisieren gereinigte Aminophenol verbraucht (im N_2-Strom) TR nur äusserst langsam. Auf Zusatz von Alkali (1 ml 2-norm. NaOH auf 7,5 mg Aminophenol) ergab die Titration mit TR bei 20° einen Verbrauch von 0,13 Mol. En-oxy-amin per Mol. Sbst.

Die Jod-Titration gibt über das Verhalten der En-amino-Oxy-Gruppe keinen Aufschluss, da der grösste Teil des Jods in den Benzolring eintritt.

6-*Hydroxy-3-amino-α-naphtoxindol* soll nach W. LANGENBECK[3] ein wirksamer Katalysator der Brenztraubensäure-Spaltung sein, und wird von ihm als »Enzym-

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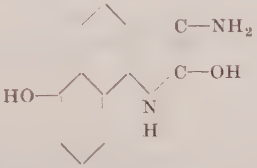
[1] Entgegen mehrfachen Angaben der Litteratur besitzt Scorbaminsäure nach Versuchen von MICHEEL keine antiskorbutischen Wirkungen.

[2] Wir sind Herrn Prof. Dr. A. BUTENANDT für die freundliche Überlassung eines Oxy-Kynurenin-Präparates sehr zu Dank verpflichtet.

[3] W. LANGENBECK, Ergebn. d. Enzymforsch. 2, 314 (1933). — Die organischen Katalysatoren. Berlin, Springer 1935. Seite 37.

Modell» der Carboxylase bezeichnet. Nach welchem Mechanismus dieser Stoff mit der Brenztraubensäure reagiert steht noch nicht fest.[1] Seit Veröffentlichung des Langenbeck'schen Enzym-Modells ist die Struktur der Auhagen'schen Cocarboxylase als Aneurin-pyrophosphat von K. Lohmann[2] aufgeklärt worden,



und Karrer und Viscontini[3] konnten eine Co-decarboxylase synthetisch darstellen, nachdem Gunsalus und Bellamy[4] nachgewiesen hatten, dass ein Phosphorsäure-ester des Pyridoxals als Co-Enzym von Aminosäure-decarboxylasen funktioniert. Strukturelle Beziehungen dieser Co-Enzyme zu den Langenbeck'schen Modellen sind nicht erkennbar.

Wie aber aus der obigen Formel ersichtlich kommt der Enolform des 6-Hydroxy-3-amino-α-naphtoxindols, wie auch den Enolformen anderer Amino-oxindole die Struktur eines Amino-reduktons zu. Man kann also annehmen, dass dieser Katalysator (unabhängig davon, ob Beziehungen zu den natürlichen Co-Enzymen bestehen) durch seine Reduktonwirkung die Decarboxylierung, welche ja eine Oxydo-reduktion darstellt, vermittelt. Dabei dürfte, wie bei der Reduktionswirkung des Pyrocatechins (und des Hydrochinons) ein Zwischenprodukt in den Reaktionsmechanismus eingreifen.

Endiamine

In dieser Klasse von Reduktonen ist zunächst das von Micheel[5] dargestellte *3,4-Diamino-tetron* zu erwähnen.

Das einfachste *aromatische Endiamin*, das *o-Phenylen-diamin*, kombinierten Euler und Hasselquist[6] mit oxydiertem Redukton (Mesoxal-dialdehyd) zu Chinoxalin-2-aldehyd. Gegen die starke Tendenz der beiden o-gestellten Aminogruppen tritt die Reduktionsfähigkeit der aromatischen Endiamine zurück.

Eine der erwähnten Chinoxalin-Bildung des o-Phenylen-diamins analoge Reaktion tritt bei den 2,4,5-Triamino-pyrimidinen ein, wobei Pteridin-derivate entstehen.

2,4,5-Triamino-6-oxy-pyrimidin steht zu den Reduktonen in zweifacher Weise in naher Beziehung:

Mit den Aminogruppen 4 und 5 dieses Stoffes reagiert Triose-Redukton selbst, ferner aber Redukton-p-Aminobenzoesäure sowie dessen Glutamat unter Bildung von Pteroyl-glutamaten, und hat dadurch zur Synthese von Folinsäure Anwendung gefunden.[1] Die Reaktion von 2,4,5-Triamino-6-oxy-pyrimidin mit Zukkern, und zwar sowohl mit Aldosen als Ketosen zu Verbindungen vom Pteridin-Typus haben KARRER und Mitarb.[2] beschrieben und haben die Struktur der dabei entstehenden Pteridine aufgeklärt.[3]

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[1] Einen Erklärungsversuch gab H. Weil-Malherbe, Nature, 145, 106 (1940). G. Barron, Ann. Rev. Biochem. 10, 1 (1941).

[2] K. Lohmann und Schuster, Biochem. Zs. 294, 188 (1937).

[3] P. Karrer und M. Viscontini, Helv. 30, 52 (1946).

[4] I. C. Gunsalus und W. D. Bellamy, J. Biol. Chem. 155, 357, 557, 685 (1944).

[5] F. Micheel, Bode und Siebert, Chem. Ber. 70, 1862 (1937).

[6] Euler und H. Hasselquist, Dieses Arkiv, 26 B, No. 5 und 26 A No. 17 (1948).

Titration des 2,4,5-Triamino-6-oxy-pyrimidins[4] mit Tillmans Reagenz

A. In neutraler Lösung (pH = 7) 20°.

5,6 mg Sbst. wurden in 10 ml Wasser aufgeschlämmt und unter Einleiten von Stickstoff mit einer 0,01 norm. Tillmans-Lösung bis zur Entfärbung titriert. Verbrauch (unvollständig): 1,65 ml TR-Lös.

Verbrauch: Gefunden 1,65 ml TR-Lös.
für 1 Mol. berechnet 7,95 ml TR-Lös.

Demgemäss ist nur 0,21 Mol. En-diamin bezw. En-oxyamin in Reaktion getreten.

B. In alkalischer Lösung (20°).

Wurde die wie oben titrierte Lösung mit 0,5 ml einer 2-norm. Natronlauge versetzt, so konnte die Titration weiter geführt werden.

Verbrauch: Gefunden 7,55 ml TR-Lös.
für 1 Mol. berechnet 7,95 ml TR-Lös.

Bei alkalischer Behandlung tritt also rund 1 Mol. En-diamin resp. En-oxyamin in Reaktion.

En-dithiole

Das Verhalten *aliphatischer* En-dithiole zu Oxydationsmitteln soll in nächster Zeit experimentell bearbeitet werden.

Von aromatischen En-dithiolen sei hier nur das Dithiobrenzcatechin erwähnt, welches schon vor langer Zeit von J. POLLAK[5] als eine *äuserst leicht oxydable Substanz* beschrieben wurde. POLLAK hat mit Wasserstoffsuperoxyd aus Dithiobrenzcatechin einen gelblichweissen Niederschlag von noch nicht aufgeklärter Struktur erhalten. Das Dithiobrenzcatechin ist nur durch einige Derivate (Dimethyläther, Diacetylderivat) charakterisiert.

Enole

Acetol (I) wird in alkalischer Lösung enolisiert, und zwar, wie wir fanden[6] zum Anion des Methyläthendiols

---

[1] R. B. ANGIER et al. (Lederle Div., Am. Cyanamide Comp.) J. Am. Chem. Soc. 70, 25 und 3029 (1948).

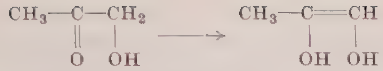
[2] P. KARRER, R. SCHWYZER, B. ERDEN und A. SIEGWART, Helv. 30, 1031 (1947).

[3] P. KARRER und R. SCHWYZER, Helv. 32, 423 (1949).

[4] Wir verdanken das hier verwendete Präparat Herrn Professor Dr. P. KARRER.

[5] J. POLLAK, Wien. Sitz. Ber. Math.-Naturwiss. Klasse. Abt. II b 122, 915 (1913).

[6] EULER und H. HASSELQUIST, Dieses Arkiv 1, No. 30 (1949).

(I)  (II)

$$CH_3{-}\underset{\underset{O}{\|}}{C}{-}\underset{\underset{OH}{|}}{CH_2} \longrightarrow CH_3{-}\underset{\underset{OH}{|}}{C}{=}\underset{\underset{OH}{|}}{CH}$$

Durch Oxydation mit Tillmans Reagenz soll dieses Methylglyoxal liefern.

G. Schwarzenbach und Ch. Wittwer[1] haben den Enol-Gehalt einfacher Ketone mit der von ihnen ausgearbeiteten bromometrischen Methode,[2] und zwar auch denjenigen des *Acetons* gemessen und berechnet; sie fanden einen prozentischen Enolgehalt von $2{,}5{,}10^{-4}$.

0,2 ml analysenreines Aceton in 10 ml Wasser + 10 ml 2-norm. NaOH unter Stickstoff entfärbte in 30 Min. bei 20° (pH = 10) einen Tropfen 0,01 norm. TR-Lösung *nicht*.

Ein Versuch bei 60° ergab unter gleichen Bedingungen ebenfalls (in alkalischer Lösung) keine Entfärbung der TR-Lösung, sondern nur einen Umschlag der blauen Farbe in rotviolett.

In Zusammenhang mit obigem Versuch über das Verhalten von Aceton zum Dichlorphenolindophenol seien auch einige, im wesentlichen negative Ergebnisse über die Oxydation von enolisierten β- und γ-Diketonen mitgeteilt Sowohl bei Acetylaceton als bei Acetonylaceton war in NaOH der Verbrauch an TR minimal.

Di-benzoyl-methan wurde dargestellt über Acetophenon-O-benzoat nach Claisen und Haase (Chem. Ber. 36 3674). Bei der Darstellung von Dibenzoylmethan durch Umlagerung von Acetophenon-O-benzoat tritt eine Enolisierung ein. Zugesetztes Tillmans-Reagenz wird entfärbt; die Menge des gebildeten Dienols bezw. dessen Oxydstionsprodukter konnte aber nicht ermittelt werden, da die Vollständigkeit des Überganges Acetophenon-benzoat Dibenzoylmethan nicht bekannt ist.

Zur Kenntnis des Triose-Reduktons

1. Erhitzen in Äthanol

Schon durch seine Aldehydgruppe und noch mehr auf Grund der Doppelbindung könnte bei Triose-Reduktion eine erhebliche Neigung zur Polymerisation erwartet werden. Besonders die Frage, wie sich die Endiolgruppe in dieser Hinsicht verhält schien von wesentlichem reaktionskinetischen Interesse zu sein. Wir haben deswegen folgenden orientierenden Versuch angestellt:

100 mg reines Redukton wurde in 100 ml 96 %igem Äthylalkohol in einem Bombenrohr 24 Stunden auf 100° erhitzt. Nach Abkühlen wurde die Reaktionsmasse im Vacuum zur Trockne eingedunstet. Es blieb ein braunes Harz zurück, das Tillmans Reagenz (TR) nicht mehr reduzierte.

Molekulargewichts-Bestimmung durch Gefrierpunktserniedrigung:

18,8 mg des obigen Produktes konnten in 2 ml Eisessig fast vollständig gelöst werden. Die Gefrierpunktserniedrigung betrug 0,275°. Daraus berechnet sich das Mol. Gew. M zu

$$\frac{18{,}8 \cdot 3900}{2108 \cdot 0{,}275} = 126.$$

Für dimerisiertes Redukton berechnet sich das Mol. Gew. 176. Das Produkt, über dessen Einheitlichkeit wir keinen Anhaltspunkt haben, war also sicher nicht vollständig dimerisiert.

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[1] G. Schwarzenbach und Ch. Wittwer, Helv. 30, 669 (1947).

[2] G. Schwarzenbach und E. Felder, Helv. 27, 1701 (1944).

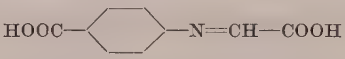
2. Oxydation von p-Aminobenzoesäure-Redukton und Isolierung des Oxydationsproduktes

Tillmans Reagenz wird durch p-Aminobenzoesäure-Redukton[1] rasch entfärbt und letzteres wird als dadurch oxydiert. Da die Reinigung und Isolierung des Oxydationsproduktes aber durch die Gegenwart des Leuko-Dichlorphenol-indophenols erschwert wird, haben wir die Darstellung des Oxydationsproduktes mittels Wasserstoffsuperoxyd durchgeführt.

1,75 g des p-Abs-Reduktons wurden in 15 ml Wasser aufgeschlämmt und mit 10 ml einer 2-norm. Natronlauge versetzt, wodurch alles mit rotbrauner Farbe in Lösung ging. Nun wurden unter ständigem Umrühren tropfenweise 2 ml einer 30 %igen H_2O_2-Lösung zugesetzt, wobei das Reaktionsgemisch sich erwärmt. Nach Abkühlung und $\frac{1}{2}$-stündigem Stehen wurde mit 1 ml Essigsäure angesäuert. Nach einiger Zeit fiel das Reaktionsprodukt in Form kleiner gelber Nadeln aus, welche in Lösung deutlich grün fluorescierten. Dieselben wurden abfiltriert und mit Wasser und Alkohol gewaschen. Sie wurden durch Auflösen in Alkali und Fällen mit Essigsäure gereinigt und schmolzen dann bei 237° unter Zersetzung.

Die Ausbeute an Reaktionsprodukt war bei wiederholten Versuchen wechselnd zwischen 0,3 und 0,85 g, entsprechend der verschiedenen oxydativen Wirksamkeit des Wasserstoffsuperoxyds.

Das Reaktionsprodukt, dem wir auf Grund des N-Gehalten und des Titrationswertes vorläufig die Zusammensetzung $C_9H_7O_4N$ und die Konstitution



zuschreiben, ist in allen üblichen organischen Lösungsmitteln schwerlöslich, ein wenig löslich in siedendem Wasser.

Analyse: Ber. für $C_9H_7O_4N$ N = 7,31 %. — Gef. N = 7,73 %.

Titration mit NaOH: 6,7 mg Sbst. in Wasser aufgeschlämmt, verbrauchten 0,68 ml 0,1 norm. NaOH.

$E_{gef} = 6{,}7 : (0{,}62\ 0{,}1) = 108$. — E_{ber} für $C_9H_7O_4N = 97$.

3. Oxydation von Sulfanilsäure-Redukton[2]

Im Gegensatz zu p-Aminosäure-Redukton liess sich die Verbindung Sulfanilsäure Redukton nicht durch Jod oxydieren. Es wurden deshalb Oxydationsversuche mit Wasserstoffsuperoxyd ausgeführt.

Die heiss gesättigte Lösung von Sulfanilsäure-Redukton wurde mit einem Überschuss von H_2O_2 versetzt. Nach 6-stündigem Stehen bei Zimmertemperatur konnte mit Aceton ein hellgelbes Produkt ausgefällt werden, das TR nicht mehr entfärbte. Durch Umkrystallisieren des Produktes aus Wasser wurde es in Form gelb glänzender Blättchen erhalten, welche über 310° schmolzen. Dieses Oxydationsprodukt ist löslich in Wasser und in warmem Pyridin, unlöslich in den gewöhnlichen organischen Lösungsmitteln.

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[1] Darstellung des p-Aminobenzoesäure-Reduktons: siehe EULER und HASSELQUIST, Rec. Trav. Chim. Pays-Bas 69 (Wibaut-Heft) 402 (1950). Zusammensetzung in Übereinstimmung mit ANGIER et al. (J. Am. Chem. Soc. 70, 25 (1948) sowie mit BELL, COCKER und O'MEARA, Biochem. J. 45, 373 (1949) 1 Mol. p-Aminobenzoesäure auf 1 Mol. Redukton.

[2] Darstellung von Sulfanilsäure-Redukton: Siehe EULER, HASSELQUIST und GLASER, Dieses Arkiv 2 (1950). In manchen Fällen erhielten wir Produkte einer weiter gehenden Oxydation bei welcher die Aldehydgruppe zu einer Carboxylgruppe oxydiert war.

Titration mit Alkali. 15,7 mg Sbst. in Wasser gelöst verbrauchten 0,78 ml einer 0,085 norm. NaOH-Lösung.

$$M = 15{,}7 : (0{,}78 \cdot 0{,}085) = 237.$$

Für $C_9H_7O_5NS$ M ber. = 241. Die Substanz wird weiter untersucht.

Stockholm, Universität, Vitamin-Institut und Inst. f. organ. chem. Forsch.

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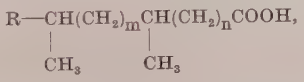
Optically active higher aliphatic compounds. VII

The synthesis of (−)-3(L),6(D)-dimethyltetracosanoic and L(+)-6-methylpentacosanoic acids

By STINA STÄLLBERG-STENHAGEN

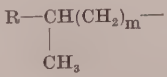
VAN'T HOFF's principle of optical superposition (1, 2) applies strictly only to stereoisomers which differ solely in the configurations of the asymmetric carbon atoms. To calculate the optical rotation of a long chain acid with several active centres by adding the latter's several contributions, on data obtained from monosubstituted acids with the active centre in the appropriate position, as was done in Part V (3), extends the principle to compounds that are structurally different — as has been done previously in other fields. The isorotation rules for sugars of C. S. HUDSON (4) represent such an extension.

If we consider a long chain acid with two active centres, *e.g.*



$$\underset{\displaystyle CH_3}{R\text{—}CH}(CH_2)_m\underset{\displaystyle CH_3}{CH}(CH_2)_nCOOH,$$

it seems likely that the rotation of the active centre nearest to the carboxyl group would be little affected if we substitute the radical



$$R\text{—}\underset{\displaystyle CH_3}{CH}(CH_2)_m\text{—}$$

for a long normal hydrocarbon chain, provided that m is large enough. Analogous considerations apply to the other active centre. The results obtained in Part V (3) for the dextrorotatory diastereoisomeric 2,9-dimethyltetracosanoic acids provide an experimental support for the above view. However, when m becomes small, *i.e.* when the active centres lie close together, deviations from the simple addition rule might be expected. For the knowledge of the relation between optical rotation and structure of long chain compounds it is, of course, important to determine the occurrence and magnitude of such deviations.

An approach has been made towards this problem by the synthesis of a long chain acid of the type depicted above, in which m is equal to two, *viz.* *(—)-3*(L),*6*(D)-*dimethyltetracosanoic acid* (VI, cf. Chart I). This acid could be prepared in a fairly straightforward manner, starting from the laevorotatory 3,6-dimethylsuberic acid obtained in the course of the work described in Part VI (5). The lengthening of the chain of the half-ester (I) of the latter acid could of course be performed by *e.g.* the β-keto ester method, but it was decided to attempt the chain-lengthening in one operation by the mixed electrolysis of the sodium salts of (I) and stearic acid in methanol solution. At first sight it might appear that the separation of the desired acid (VI) from the other products of the electrolysis would prove very difficult. However, the solubility properties of the compounds involved are favourable in this case. The long chain hydrocarbon (*n*-tetratriacontane (IV)) produced is nearly insoluble in the electrolysis mixture, and most of it can be removed by simple filtration. The removal of the dibasic acid (VII) after the hydrolysis of the mixture of the methyl esters (III) and (V) formed in the electrolysis was not difficult, as (VII) was found to be extremely soluble in all organic solvents tried, with the exception of low-boiling light petroleum. The overall yield of the desired acid (VI) was 14 % of the theoretical, calculated on (I). Although the yield is rather low, the chain-lengthening method used has the advantage of simplicity.

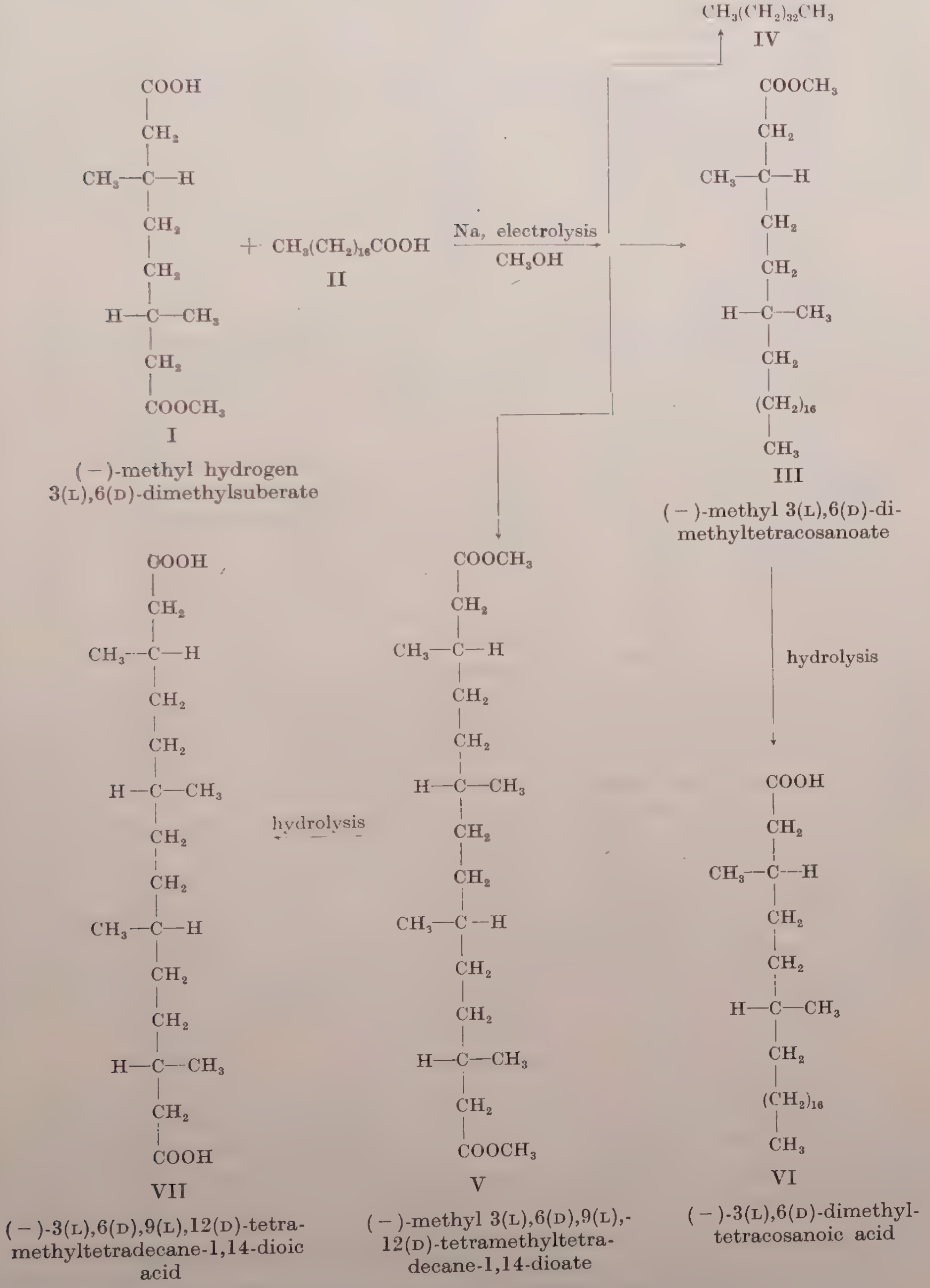
(—)-3(L),6(D)-Dimethyltetracosanoic acid, $[\alpha]_D^{21} -3.92°$, $[M]_D^{21} -15.6°$, has three different crystalline forms. The acid is obtained from light petroleum in the form of long thin needles which on heating undergo a transition at 41° to a second form, which melts at 45.0–45.2°. The latter form is also obtained by crystallization from glacial acetic acid. A third, metastable form can be obtained by solidification of the melt. This form melts at 34.0–34.5°. The *amide* is dimorphic, the two forms melting at 83.9–84.2° and 88.4–88.5° respectively.

The dibasic acid (VII) could not be isolated in a pure form from the mixed electrolysis, and in order to study its properties a specimen of the methyl ester (V) was prepared by electrolysis of the sodium salt of (—)-methyl hydrogen 3,6-dimethylsuberate (I). (—)-*Methyl 3*(L),*6*(D),*9*(L),*12*(D)-*tetramethyltetradecane-1,14-dioate* (V) has $[\alpha]_D^{20} -7.02°$, $[M]_D^{20} -24.1°$ (homogeneous). Hydrolysis of the ester gave the corresponding *acid* (VII) which melts at 34–35° and has $[\alpha]_D^{21} -7.6°$, $[M]_D^{21} -24°$. (—)-*3*(L),*6*(D),*9*(L),*12*(D)-*Tetramethyltetradecane-1,14-diamide* melted at 123.2–123.8°.

In order to compare the observed rotation of the 3,6-dimethyl substituted acid with that calculated from the rotations of 3-methyl- and 6-methyl substituted acids it became necessary to synthesize a long chain acid of the latter type, as the rotation of 6-methyl substituted acids of high molecular weight was not previously known. The synthesis of L(+)-*6-methylpentacosanoic acid* (XIV) was carried out in the manner outlined in Chart II, starting from L(—)-*3-methyldocosanoic acid* (VIII). The latter acid was prepared by the method described in Part II (6). The intermediate L(+)-*3-methyl-5-ketodocosanoic acid* had $[\alpha]_D^{22} +1.35°$, $[M]_D^{22} +5.0°$. The keto-acid can exist in two crystalline forms which melt at 66.8–67.1° and 67.8–68.2° respectively.

L(—)-3-Methyldocosanoic acid (VIII), $[\alpha]_D^{21} -3.95°$, $[M]_D^{21} -13.4°$, m.p. 58.1–58.3°, was converted into L-*methyl 3-keto-5-methyltetracosanoate* (XI) using the usual procedure, the yield being 61 % of the theoretical. The β-keto ester has two crystalline modifications melting at 49.8–50.5° and 39.6–40.0° respectively. Alkylation of the β-keto ester (XI) by means of ethyl chloroacetate was carried out under the usual conditions. Hydrolysis and ketonic cleavage of the alkylation product (XIII) gave L(—)-*4-keto-6-methylpentacosanoic acid* (XIV) in a yield of 35 %, calculated on (XI). This keto-acid has an optical rotation $[\alpha]_D^{23} -4.4°$ ($[M]_D^{23} -18.2°$) and has two crystalline modifications which melt at 73.5° and 78.7–79.1° respectively. Reduction of the keto-acid by CLEMMENSEN's method in ethanol solution, followed by hydrolysis of the ethyl ester formed during the reduction, finally gave L(+)-6-methylpentacosanoic acid (XV). The yield of reduced acid was 72 % of the theoretical. The 6-

Chart I.[1]


$CH_3(CH_2)_{32}CH_3$
IV
+ $CH_3(CH_2)_{16}COOH$
II
Na, electrolysis
CH_3OH
I
(−)-methyl hydrogen 3(L),6(D)-dimethylsuberate
III
(−)-methyl 3(L),6(D)-dimethyltetracosanoate
hydrolysis
hydrolysis
VII
(−)-3(L),6(D),9(L),12(D)-tetramethyltetradecane-1,14-dioic acid
V
(−)-methyl 3(L),6(D),9(L),12(D)-tetramethyltetradecane-1,14-dioate
VI
(−)-3(L),6(D)-dimethyltetracosanoic acid

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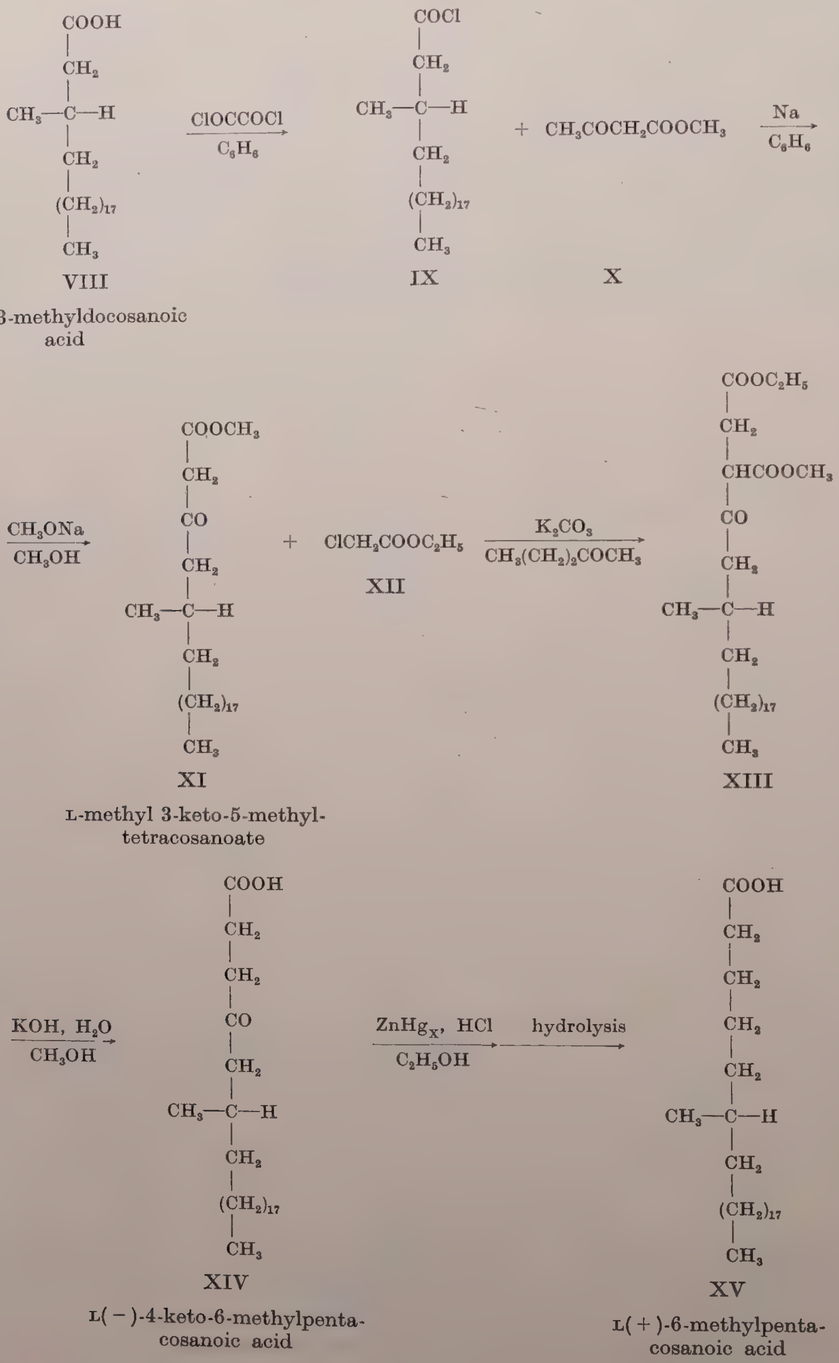
[1] The formulae are written according to the FISCHER convention.

Chart II.


ClOCCOCl
C_6H_6
+ $CH_3COCH_2COOCH_3$
Na
C_6H_6
CH_3ONa
CH_3OH
+ $ClCH_2COOC_2H_5$
K_2CO_3
$CH_3(CH_2)_2COCH_3$
KOH, H_2O
CH_3OH
$ZnHg_x$, HCl
C_2H_5OH
hydrolysis

VIII

L(−)-3-methyldocosanoic acid

IX

X

XI

L-methyl 3-keto-5-methyltetracosanoate

XII

XIII

XIV

L(−)-4-keto-6-methylpentacosanoic acid

XV

L(+)-6-methylpentacosanoic acid

methyl substituted acid was found to possess a very low optical rotation, α_D^{25} for a solution in chloroform (*l*, 1; *c*, 27) being +0.06°, corresponding to a specific rotation $[\alpha]_D^{25}$ +0.22° and a molecular rotation $[M]_D^{25}$ +0.9°. The acid is dimorphic, the two modifications melting at 53.7–54.1° and 55.6–56.0°. L(+)-*6-Methylpentacosanamide* has m.p. 90.0–90.2°. Dimorphism has not been observed for this amide.

The optical rotation of L(—)-3-methyltetracosanoic acid (Part II (6)), L(+)-6-methylpentacosanoic acid, and (—)-3(L),6(D)-dimethyltetracosanoic acid has been measured for five wavelengths in the visible and near ultraviolet, using a photoelectric ultraviolet polarimeter of the BRUHAT-GUINIER (7) type, built in this laboratory.[1] The results are collected in Table I.

Table I

Molecular rotations in chloroform solution at 25°.

Wave-length Å	L(−)-3-Methyl-tetracosanoic acid		L(+)-6-Methylpenta-cosanoic acid	(−)-3(L),6(D)-Dimethyltetra-cosanoic acid			
				Calcd		Found	
	c, 7.22	*c*, 12.03	*c*, 27				
	A	B	C	(A − C)	(B − C)	*c*, 7.39	*c*, 13.32
5893	−12.9		+0.9	−13.8		−15.1	−15.2
5461	−15.6	−14.9	+1.1	−16.7	−16.0	−18.2	−18.0
4358	−26.9	−25.6	+2.0	−28.9	−27.5	−31.3	−31.3
4047	−32.9	−31.5	+2.4	−35.3	−33.9	−37.6	−37.9
3650	−43.0	−41.7				−50.2	−50.3

The molecular rotation of the active 3-methyltetracosanoic acid varies slightly with concentration, the rotation being higher for more dilute solutions. No such variation is found for the 3,6-dimethyl substituted acid. Owing to the small rotation, the concentration dependence of the molecular rotation of the 6-methyl substituted acid could not be measured with any degree of accuracy. Table I shows that the experimentally found molecular rotation of (—)-3(L),6(D)-dimethyltetracosanoic acid is slightly higher than that calculated by adding the molecular rotations of L(—)-3-methyltetracosanoic acid and D(—)-6-methylpentacosanoic acid. However, the difference is of the same order of magnitude as the concentration effect, and it is evident that, even when the active centres are separated by only two CH_2 groups ($m = 2$), the simple addition rule allows the calculation of the molecular rotation of the molecule with a fair degree of accuracy.

Experimental[2]

(—)-*Methyl hydrogen 3*(L),*6*(D)-*dimethylsuberate* (I). (—)-3(L),6(D)-Dimethylsuberic acid (Part VI (5)) (50.5 g, 0.25 mole, $[\alpha]_D^{21}$ −16.9° (chloroform; *c*, 8.41)) was partially esterified by refluxing for 5 hours with methanol (12 g, 0.375 mole) and concentrated hydrochloric acid (5 ml). Methanol and hydrochloric acid were distilled off *in vacuo* using a water pump. The residue was distilled through a 15 cm electrically heated and packed column at a pressure of 0.5 mm. The following fractions were collected.

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[1] The construction of this instrument was made possible by a grant from *Statens Tekniska Forskningsråd.*

[2] Melting points, crystal spacings and optical rotations were determined as described in Part I (9).

I 83°	17.2 g	α_D^{22} —10.71° (hom., *l*, 1)
II 83—116°	1.9 g	—
III 116—120°	24.8 g	α_D^{21} —12.30° (hom., *l*, 1)

The solid residue in the flask amounted to about 10 g. Fraction III consists of (—)-*methyl hydrogen 3*(L),*6*(D)-*dimethylsuberate.* Yield 46 % of the theoretical.

Anal.: Calcd for $C_{11}H_{20}O_4$ (261.3)	C, 61.08;	H, 9.31 %
Found	C, 60.80;	H, 9.26 %

Fraction II and the residue were combined and esterified with methanol, giving 10.4 g of dimethyl ester, b.p. 82°, 0.5 mm, α_D^{21} —10.73° (hom., *l*, 1). The total yield of dimethyl ester was thus 27.6 g, or 48.6 % of the theoretical.

n-*Octadecanoic* (*stearic*) *acid* (II). The stearic acid "pro analysi" manufactured by LKB-Produkter, Stockholm, was found to be homologue-free, but contained a small amount of unsaturated material (probably mainly oleic acid). The unsaturated material was removed by treating the commercial product with concentrated sulphuric acid at a temperature of 70°, as described by FRANCIS, KING and COLLINS (8). Considerable darkening occurred. The recovered acid was recrystallized twice from glacial acetic acid. The sulphuric acid treatment was then repeated. After two crystallizations from glacial acetic acid the stearic acid had m.p. 69.2–69.4°. FRANCIS and PIPER (10) give m.p. 69.4°.

(—)-*3*(L),*6*(D)-*Dimethyltetracosanoic acid* (III). Sodium (0.4 g) was dissolved in methanol (230 ml, LKB-Produkter "pro analysi"), and stearic acid (9.5 g, 0.033 mole) and (—)-methyl hydrogen 3,6-dimethylsuberate (7.2 g, 0.033 mole) added. The electrolysis was carried out between platinum electrodes (cf Part VI (5)), using a current of 3.5-4 amps. In order to avoid excessive foaming, the outside of the electrolysis vessel (thin-walled beaker of 400 ml capacity) was cooled by water kept at a temperature of 38°. More intensive cooling led to the separation of sodium stearate from the electrolysis mixture. Soon after the start of the electrolysis, *n*-tetratriacontane began to separate, making cleaning of the anode necessary at intervals. The electrolysis was continued until the pH of the solution had risen to 7. The mixture was filtered hot to remove the insoluble reaction products. To the filtrate were added 9.5 g of stearic acid and 7.2 g of half-ester, and the electrolysis continued until the pH of the solution again reached 7.

After cooling, the mixture was filtered, the filtrate diluted with water and extracted with ether, and the ether solution shaken with a 5 % solution of sodium carbonate. The precipitate of sodium stearate was removed, and the treatment repeated until no precipitate was obtained. After drying (sodium sulphate) the ether was distilled off and the residue (8 g) subjected to hydrolysis by means of potassium hydroxide (20 g), water (15 ml), and ethanol (200 ml of 99.5 %). The mixture was refluxed for 5 hours. In order to remove neutral products, about 125 ml of water was added to the mixture while the latter was still hot, and the resulting solution extracted twice with light petroleum (b.p. 40–60°). The alkaline water-ethanol solution was acidified by means of hydrochloric acid and extracted with ether. The ether solution was dried, the solvent distilled off, and the residue dissolved in 150 ml of methanol containing 7 % of water. After being kept at a temperature of 0° overnight the white microcrystalline material that had separated was filtered off. Yield 3.7 g of m.p. 41–42°. After crystallization from methanol some of the material melted at 41°, but the bulk of the specimen did not melt until 43.5–44.0°. After further crystallization from light petroleum (b.p. 40–60°) at 0° (—)-*3*(L),*6*(D)-*dimethyltetracosanoic acid* was obtained in the form of very thin silky needles 2–3 mm long. On heating a transition occurred at 41° with the formation of an opaque solid, which on further heating melted at 45.0–45.2°. The latter form was obtained at room temperature by crystallization from glacial acetic acid. A third crystalline form was sometimes obtained on rapid cooling of the melt. This metastable modification melted at 34.0–34.5°. On further heating resolidification with the formation of the form of m.p. 45° took place. The crystal modification obtained from light petroleum gave very good diffraction patterns with a long spacing of 47.3 Å. The microcrystalline material obtained from glacial acetic acid had a long crystal spacing of 31.7 Å. The unstable low-melting form had a somewhat translucent appearance and the diffraction pattern from a specimen in a capillary tube showed a single strong side spacing of 4.14 Å. The two other crystalline forms gave side spacing patterns consisting of a large number of lines.

Optical rotation α_D^{21} —0.94° (chloroform; *l*, 4; *c*, 5.98)
$[\alpha]_D^{21}$ —3.92° $[M]_D^{21}$ —15.6°

Anal.: Calcd for $C_{26}H_{52}O_2$ (396.7) C, 78.72; H, 13.21 %
Found C, 79.05; H, 13.11 %

(—)-*3*(L),*6*(D)-*Dimethyltetracosanamide* was prepared from 1 g of acid, with the usual procedure (cf Part I (9)). After crystallization from methanol the amide (0.68 g) was obtained as a white microcrystalline material showing a weak lustre. The m.p. was 88.2–88.5°, unchanged on further crystallization. If a sample crystallized from methanol was placed in the melting point bath at a temperature of 85° it melted partly, then solidified, and on further heating melted at 88.2–88.5°. On slow heating from room temperature only the higher m.p. was observed. On cooling of the melt solidification usually took place at about 75° with the formation of an opaque solid showing no distinct crystalline structure. This form melted at 83.9–84.2°. Sometimes solidification occurred at 87°, however, with the formation of fine needle-shaped crystals, which on reheating melted at 88.4–88.7°.

Long X-ray spacing (pressed specimen) 44.7 Å. The same spacing was also found for a melted specimen, but in both cases the diffraction patterns showed some weak extra lines.

Optical rotation α_D^{21} —0.21° (chloroform; *l*, 2; *c*, 3.61)
$[\alpha]_D^{21}$ —2.9° $[M]_D^{21}$ —11.5°

Anal.: Calcd for $C_{26}H_{53}ON$ (395.7) N, 3.54 %
Found N, 3.64 %

n-*Tetratriacontane.* The precipitate formed during electrolysis, which had been removed by filtration at the end of each run, was dissolved in ether. After keeping the solution at 0° overnight, 7.1 g of *n*-tetratriacontane crystallized out in the form of fairly large lustrous plates. The hydrocarbon melted, without further purification, at 72.7–72.8°. CHIBNALL *et al.* (11) give m.p. 72.6–72.8°.

(—)-3(L),6(D),9(L),12(D)-Tetramethyltetradecane-1,14-dioic acid (VII). The methyl ester of this dibasic acid should be formed during the electrolysis, but the dibasic acid could not be isolated in pure form after hydrolysis of the mixed methyl esters from the electrolysis. In order to study the properties of the acid a specimen was prepared in a separate experiment by electrolyzing (—)-methyl hydrogen 3(L),6(D)-dimethylsuberate (8.9 g) in a solution of sodium (0.14 g) in methanol (60 ml). The half-ester was added in two portions, and the electrolysis performed between a set of small platinum electrodes (1.5 × 2.5 cm). 2.2 Amps were run through until the pH of the solution had risen to 7 after each addition of half-ester. Water was added and the mixture extracted with ether. The ether solution was washed with sodium carbonate solution and with water, dried (sodium sulphate), and the ether distilled off. The residue was subjected to hydrolysis, and the free acid taken up in ether in the standard manner. The residue obtained after removal of the ether did not crystallize, even at low temperatures. Crystallization from solvents (methanol, light petroleum) was attempted, but met with no success. From methanol-water only oily droplets were obtained. The material was therefore esterified by refluxing with methanol (200 ml) and concentrated sulphuric acid (10 ml) for 10 hours. The neutral products from the esterification were distilled. The following fractions were collected.

I	30–90°,	0.5 mm	1 ml
II	90–146°,	0.5 »	0.5 »
III	146–148°,	0.5 »	2.74 g

Fraction III, a colourless mobile liquid, consists of (—)-*methyl 3(L),6(D),9(L),12(D)-tetramethyltetradecane-1,14-dioate* (V). Yield 39 % of the theoretical. d_4^{20} 0.940.

Optical rotation α_D^{20} —6.60° (homogeneous; *l*, 1)
$[\alpha]_D^{20}$ —7.02° $[M]_D^{20}$ —24.1°

Anal.: Calcd for $C_{20}H_{38}O_4$ (342.5) C, 70.13; H, 11.18 %
Found C, 69.85; H, 11.14 %

The ester just described (2.1 g) was hydrolyzed and the free acid taken up in ether in the usual manner. The residue obtained after the ether had been distilled off solidified in the refrigerator to a colourless crystalline solid. M.p. 34°. The dibasic acid is exceedingly soluble in most organic solvents and it failed to separate from acetone even at —70°. The acid was distilled in a high-vacuum microsublimation apparatus. The distillate, a colourless syrup, crystallized very slowly at room temperature. Cooling to low temperatures gave a solid glass. The solid that had crystallized at room temperature melted at 34–35°. The X-ray diffraction pattern of this material did not show any distinct 00*l* reflexions.

Optical rotation α_D^{21} —0.76° (chloroform; *l*, 2; *c*, 4.99)
$[\alpha]_D^{21}$ —7.6° $[M]_D^{21}$ —24°

Anal.: Calcd for $C_{18}H_{34}O_4$ (314.5) C, 68.84; H, 10.90 %
Found C, 68.90; H, 10.85 %

The *diamide* was prepared via the acid chloride in the usual manner. The compound is very soluble in methanol and ethanol, and was crystallized from acetone and from benzene or benzene-light petroleum containing a small amount of ethanol. The diamide was finally obtained as a white microcrystalline powder. M.p. 123.2–123.8°, after sintering at 121°. The m.p. was the same after previous melting.

Anal.: Calcd for $C_{18}H_{36}O_2N_2$ (312.5) N, 8.96 %
Found N, 9.04 %

L(+)-*3-Methyl-5-ketodocosanoic acid.* The keto-acid was prepared exactly as described for the higher homologue synthesized in Part II (6), using active methyl 3-keto-5-methylpimelate (35 g, prepared from D(—)-methyl hydrogen β-methylglutarate of α_D^{21} —2.65° (hom., *l*, 4) and 1-iodohexadecane (57 g). (The iodide, b.p. 149–150°, 0.7 mm, was prepared from a specimen of *n*-hexadecanol-1 that had been carefully purified via the acetate. In order to remove homologous impurities the acetate had been distilled through a 1 m electrically heated and packed column. The b.p. of the *n*-hexadecyl acetate was 155°, 1.2 mm.) After hydrolysis and ketonic cleavage of the alkylation product crystallization from ethanol gave 30.5 g of crude keto-acid. Recrystallization from light petroleum (b.p. 40–60°) gave 23 g of L(+)-*3-methyl-5-ketodocosanoic acid* in the form of very thin lustrous prisms with a length of 1–2 cm. M.p. 66.5–67.1°. After previous melting the m.p. was 66.8–67.1°. Crystallization from ethanol gave another crystalline form having m.p. 67.8–68.2°. The m.p. of a previously melted specimen was then 66.8–67.1°. The keto-acid was obtained from ethanol in the form of very small felted needles.

The crystals obtained from light petroleum gave beautiful diffraction patterns with a long spacing of 44.3 Å. Specimens prepared by the hot-wire technique gave the same diffraction patterns. A pressed specimen of the keto-acid crystallized from ethanol gave a complex diffraction pattern in which 00*l* reflexions could not be identified with certainty.

Optical rotation $\alpha_D^{22} + 0.50°$ (chloroform; *l*, 4; *c*, 9.22)
$[\alpha]_D^{22} + 1.35°$ $[M]_D^{22} + 5.0°$

Anal.: Calcd for $C_{23}H_{44}O_3$ (368.6) C, 74.94; H, 12.03 %
Found C, 74.94; H, 12.00 %

L(—)-*3-Methyldocosanoic acid* (VIII). The reduction of the keto-acid just described (19.5 g) was carried out by CLEMMENSEN's method in the manner outlined in Part II (6). Crystallization from light petroleum (b.p. below 40°) gave 13.6 g of reduced acid of m. p. 57.6–57.9°. Slow crystallization from benzene gave L(—)-*3-methyldocosanoic acid* in the form of beautiful large crystals. Single crystals having a length of about 1 cm and a cross-section of 1 × 5 mm have been obtained. These crystals melted at 58.1–58.3°. The acid was obtained from glacial acetic acid in the form of thin lustrous plates with the same m.p. Long X-ray spacing (pressed specimen) 30.6 Å.

Optical rotation $\alpha_D^{21} - 1.60°$ (chloroform; *l*, 4; *c*, 10.11)
$[\alpha]_D^{21} - 3.95°$ $[M]_D^{21} - 13.4°$

Anal.: Calcd for $C_{23}H_{46}O_2$ (354.6) C, 77.90; H, 13.08 %
Found C, 78.05; H, 13.00 %

D(+)-*3-Methyldocosanamide* was prepared via the acid chloride from 0.5 g of acid. Crystallization from benzene gave the amide in the form of very thin small plates, m.p. 98.6–99.2°. After further crystallization from methanol the amide was obtained in the form of silky needles, m.p. 98.5–99.0°. Two melting points, *viz.* 99.2–99.6° and 96.2–96.6° respectively, have been observed for previously melted specimens. Pressed specimens from methanol and from benzene, as well as a melted sample, gave a long X-ray spacing of 32.0 Å, but the diffraction patterns differed considerably in appearance.

Anal.: Calcd for $C_{23}H_{47}ON$ (353.6) N, 3.98 %
Found N, 4.00 %

L-*Methyl 3-keto-5-methyltetracosanoate* (XI). The β-keto ester was prepared from 8.53 g of L(−)-3-methyldocosanoic acid by the modified BOUVEAULT-BONGERT-HUNSDIECKER method (12), in the manner described for the lower homologue synthesized in Part V (3). Crystallization from ethanol gave 7.03 g of crude β-keto ester of m.p. 46.8–48.8°. After crystallization from acetone the m.p. was 48.6–50.0°. On immediate reheating of the solidified melt remelting occurred at 40.2–40.8°, indicating the presence of a second crystalline form. The yield of material with the m.p.'s just recorded was 6.07 g or 61 % of the theoretical. Further crystallization from ethanol gave L-*methyl 3-keto-5-methyltetracosanoate* in the form of very small lustrous plates, m.p. 49.8–50.5°. Previously melted material had m.p. 39.6–40.0°. The low-melting form is stable only near the m.p., and previously melted material that had been kept at room temperature remelted at 50.5°.

Long X-ray spacing (high-melting form) 29.1 Å.

Optical rotation α_D^{22} −1.43° (chloroform; *l*, 4; *c*, 10.23)

Anal.: Calcd for $C_{26}H_{50}O_3$ (410.7) C, 76.04; H, 12.27 %
Found C, 76.10; H, 12.26 %

L(−)-*4-Keto-6-methylpentacosanoic acid* (XIV). L-Methyl 3-keto-5-methyltetracosanoate (5 g), ethyl chloroacetate (1.5 g), dry potassium carbonate (6 g), and dry methyl-*n*-propyl ketone (20 ml) were boiled under reflux for 18 hours. The mixture, which had turned yellow-brown, was worked up in the standard manner. Hydrolysis and ketonic cleavage of the alkylation product (XIII) was carried out by means of potassium hydroxide (10 g) in water (10 ml) and methanol (150 ml). The mixture was kept at a temperature of 50° overnight. The solution was acidified and extracted with ether in the usual manner. The residue obtained after removal of the ether was crystallized from light petroleum (b.p. 40–60°), giving 2.44 g of a yellow-white microcrystalline powder of m.p. 70–72°. The crude keto-acid was dissolved in acetone, charcoal added, and the mixture boiled for two minutes and filtered while still hot. On cooling, L(−)-*4-keto-6-methylpentacosanoic acid* was obtained in the form of small lustrous plates, which melted at 78.6–79.0°. After previous melting a part of the material that had been deposited on the side of the melting point tube melted at 73°, but the greater part of the material melted at 78.8–79.1°. A second lot of crude acid treated in the same manner gave the purified keto-acid in a microcrystalline form of m.p. 73.5°, evidently corresponding to the form observed for a part of the previously melted material referred to above. After melting resolidification took place and on further heating final melting occurred at 78.7–79.1°.

Long X-ray spacings 49.5 (high-melting form) and 45.3 Å (low-melting form).

Optical rotation $\alpha_D^{23} -0.80^\circ$ (chloroform; *l*, 4; *c*, 4.49)
$[\alpha]_D^{23} -4.44^\circ$ $[M]_D^{23} -18.2^\circ$

Anal.: Calcd for $C_{26}H_{50}O_3$ (410.7) C, 76.04; H, 12.27 %
Found C, 75.84; H, 12.16 %

L(+)-*6-methylpentacosanoic acid* (XV). The keto-acid just described (1.34 g) was reduced by CLEMMENSEN's method in ethanol solution, using the standard procedure. After hydrolysis of the ethyl ester formed during the reduction the acid was crystallized twice from light petroleum (b.p. 40–60°). L(+)-*6-methylpentacosanoic acid* (0.93 g) was obtained in the form of very small plates of m.p. 53.7–54.1°. The m.p. was unchanged after previous melting. Crystallization from glacial acetic acid gave a second crystalline form (microcrystalline) of m.p. 55.6–56.0°. Recrystallization of this material from light petroleum gave the form of m.p. 53.7–54.1°.

Long X-ray spacings 50.6 (low-melting form) and 34.1 Å (high-melting form).

Optical rotation $\alpha_D^{22} +0.02^\circ$ (chloroform; *l*, 2; *c*, 4)
$[\alpha]_D^{22} +0.25^\circ$ $[M]_D^{22} +1^\circ$

Anal.: Calcd for $C_{26}H_{52}O_2$ (396.7) C, 78.72; H, 11.84 %
Found C, 78.65; H, 11.80 %

L(+)-*6-Methylpentacosanamide*. The amide was prepared via the acid chloride from 0.13 g of acid. After crystallization from benzene the microcrystalline product exhibited a weak lustre. The m.p. was 89.9–90.1°, unchanged after previous melting. After recrystallization from ether the m.p. was 90.0–90.2°.

Long X-ray spacing (pressed specimen) 50.3 Å.

Anal.: Calcd for $C_{26}H_{53}ON$ (395.7) N, 3.54 %
Found N, 3.58 %

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SUMMARY

The synthesis of (—)-3(L),6(D)-dimethyltetracosanoic acid through mixed electrolysis of the sodium salts of (—)-methyl hydrogen 3(L),6(D)-dimethylsuberate and stearic acid in methanol solution is described. In order to compare the optical rotation of this acid with that calculated by adding the rotations of the appropriate 3- and 6-methyl substituted acids, L(+)-6-methylpentacosanoic acid has been synthesized from L(—)-3-methyldocosanoic acid via the β-keto ester method.

The optical rotations of L(—)-3-methyltetracosanoic acid, L(+)-6-methylpentacosanoic acid, and (—)-3(L),6(D)-dimethyltetracosanoic acid have been measured for five wavelengths in the visible and near ultraviolet. It is found that the mole-

cular rotation of the dimethyl substituted acid can be calculated with a fair degree of accuracy by adding the molecular rotations of the appropriate monomethyl substituted acids.

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A comparison between the potassium content of unfertilized and fertilized sea-urchin eggs

By Mignon Malm and Louise Wachtmeister

Runnström (1930, 1933 A, 1933 B) has demonstrated that acid formation accompanies the fertilization of the eggs of *Paracentrotus lividus.* The acid formation is limited to the first fifteen to twenty minutes after fertilization. These results have been confirmed by other authors on the same species as well as on *Psammechinus miliaris* (Borei 1933, Laser and Rotschild 1939). The chemical nature of the acid formation is, however, still unknown.

Another change demonstrated in the sea-urchin egg upon fertilization is a sudden breakdown of carbohydrates (Örström and Lindberg 1940).

In earlier investigations (Malm 1940, 1945, 1947) we found that certain weak acids cause a potassium loss upon penetrating the yeast cell. At the same time carbohydrates seem to break down. It was considered of interest to examine if the potassium loss and the breakdown of carbohydrates were caused by the hydrogen ions which appear in the cell when the weak acid partly dissociates in the cell.

In this connection, experiments were instigated to determine if the acid formation in the sea-urchin egg were accompanied by a loss of potassium from the cell when the carbohydrate breakdown occurres. A comparison was therefore made between the potassium content of unfertilized and fertilized eggs of sea-urchins. Two species were examined: *Strongylocentrotus droebachiensis* and *Psammechinus miliaris.*

Experimental

The eggs were washed several times in a centrifuge in surface sea-water. They were then suspended in sea-water to which glycine had been added to the extent of 0.1 %. This suspension was divided into two equal parts, the one of which was fertilized. The pH of the sea-water was adjusted to 8.2. The glycine was added in order to make the fertilization as complete as possible. Upon an average 92 % of the eggs became fertilized in this way.

A certain time after fertilization the fertilized, as well as the unfertilized, eggs were washed three times in a centrifuge with potassium-free artificial sea-water. This water was prepared according to Öhman (1944) where KCl was replaced by a corresponding amount of NaCl. The salt content of this sea-water was 32 %. For our purposes, however, it was diluted to 25 % and alkalized to pH 8.2.

The material was immediately frozen at —15° C and dried afterwards in this state. The material was then combusted by the wet method (H_2SO_4 and H_2O_2). Analyses were carried out for potassium, phosphorus and nitrogen on the combusted samples.

Table

The content of some ions in the egg of *Psam-*

1. No.	2. Time in min. after fertilization	3. Mg. K/100 mg. dried eggs	4. % difference in K between unfert. and fert.	5. Mg. N/100 mg. dried eggs	6. % difference in N between unfert. and fert.
12.7. 49.					
Unfertilized	15	3.21	—1.0	9.47	+ 1.2
Fertilized	»	3.18		9.58	
13.7. 49.					
Unfertilized	20	3.13	—8.0	9.78	— 8.8
Fertilized	»	2.88		8.92	
14.7. 49. I.					
Unfertilized	20	3.01	+1.3	9.33	— 1.2
Fertilized	»	3.05		9.22	
14.7. 49. II.					
Unfertilized	20	3.08	±0	9.72	— 2.7
Fertilized	»	3.08		9.46	
15.7. 49. I.					
Unfertilized	20	3.27	—4.3	9.92	— 6.5
Fertilized	»	3.13		9.28	
15.7. 49. II.					
Unfertilized	20	3.23	—5.3	9.81	— 6.6
Fertilized	»	3.06		9.16	
16.7. 49. I.					
Unfertilized	20	3.19	—4.1	9.40	— 3.0
Fertilized	»	3.06		9.12	
16.7. 49. II.					
Unfertilized	10	3.08	—3.2	9.52	—12.8
Fertilized	»	2.98		8.30	
17.7. 49.					
Unfertilized	18	3.56	—8.1	9.65	— 8.8
Fertilized	»	3.27		8.80	
18.7. 49.					
Unfertilized	10	3.54	—4.5	9.51	— 0.2
Fertilized	»	3.38		9.49	
19.7. 49. I.					
Unfertilized	50	3.51	—2.8	9.61	— 2.7
Fertilized	»	3.41		9.35	
19.7. 49. II.					
Unfertilized	40	3.57	—5.9	9.85	— 8.7
Fertilized	»	3.36		8.99	
Average					
Unfertilized		3.29	—4.3	9.63	— 5.1
Fertilized		3.15		9.14	

echinus miliaris before and after fertilization.

7. Mg. P/100 mg. dried eggs	8. % difference in P between unfert. and fert.	9. Mg. Cl/100 mg. dried eggs	10. Increase in Cl in % after fertiliz.	11. Mg. Na/100 mg. dried eggs	12. Increase in Na in % after fertiliz.
1.34 1.23	— 8.2	5.25 4.95	— 9.4	3.46 3.28	— 5.2
1.43 1.42	— 0.7	2.98 5.76	+ 93.3	2.07 3.87	+87.0
1.41 1.38	— 2.1	3.11 3.16	+ 98.1	2.03 4.03	+98.5
1.43 1.48	+ 3.4	3.89 4.95	+ 27.3	2.63 3.20	+21.7
1.53 1.43	— 6.5	3.12 6.54	+109.6	2.03 4.01	+97.5
1.49 1.34	—10.1	3.32 5.42	+ 63.2	2.32 3.87	+66.8
1.42 1.32	— 7.0	4.31 6.40	+ 48.5	2.90 3.94	+35.9
1.42 1.34	— 5.6	3.84 6.58	+ 71.4	2.68 4.44	+65.7
1.49 1.34	—10.1	4.45 8.36	+ 87.9	2.95 4.82	+61.2
1.42 1.35	— 4.9	4.10 5.40	+ 31.7	2.68 3.49	+30.2
1.48 1.41	— 4.7	3.37 4.73	+ 40.4	2.18 3.22	+47.7
1.57 1.40	—10.8	2.96 5.94	+100.7	2.05 3.96	+93.2
1.45 1.37	— 5.6	3.73 5.93	+ 59.0	2.50 3.84	+58.4

Table

The content of some ions in the egg of *Strongylo-*

1. No.	2. Time in min. after fertilization	3. Mg. K/100 mg. dried eggs	4. % difference in K between unfert. and fert.	5. Mg. N/100 mg. dried eggs	6. % difference in N between unfert. and fert.
4.3. 49.					
Unfertilized	3/4	2.44	−6.1	8.55	−10.0
Fertilized	»	2.29		7.70	
5.3. 49.					
Unfertilized	3/4	2.44	−12.3	8.47	−6.3
Fertilized	»	2.14		7.94	
26.2. 49.					
Unfertilized	1 1/4	2.38	−1.7	8.29	−8.3
Fertilized	»	2.34		7.60	
1.3. 49.					
Unfertilized	1 1/4	2.51	−3.6	8.46	−6.6
Fertilized	»	2.42		7.90	
3.3. 49.					
Unfertilized	1 1/4	2.51	−5.2	8.54	−10.4
Fertilized	»	2.38		7.65	
9.3. 49.					
Unfertilized	2	2.52	−7.1	—	—
Fertilized	»	2.34		—	
10.3. 49.					
Unfertilized	4	2.54	−8.7	8.40	−9.4
Fertilized	»	2.32		7.61	
Average					
Unfertilized		2.48	−6.4	8.45	−8.5
Fertilized		2.32		7.73	

Potassium and sodium were determined with the aid of the BECKMAN flame spectrophotometer. The phosphorus analyses were performed by BRIGGS' (1924) colorimetric method and the nitrogen analyses according to the modified method of KJELDAHL by STOVER and SANDIN (1931). Chloride was determined according to REHBERG's micro-method as modified by SCHNOHR (see KROGH 1938). In this case the eggs were combusted with the aid of nitric acid in the presence of $AgNO_3$.

Results

Experiments with Psammechinus miliaris

The eggs begin to divide about 50–55 minutes after fertilization. The acid formation occurres during the first 20 minutes after fertilization. Therefore, samples were taken within this time. The results of 12 experiments are given in Table 1.

entrotus droebachiensis before and after fertilization.

7. Mg. P/100 mg. dried eggs	8. % difference in P between unfert. and fert.	9. Mg. Cl/100 mg. dried eggs	10. Increase in Cl in % after fertiliz.	11. Mg. Na/100 mg. drieds eggs	12. Increase in Na in % after fertiliz.
1.03 0.94	−9.1	3.26 6.48	+ 8.8	2.24 4.38	+ 95.5
1.02 1.01	−1.0	3.33 5.42	+ 2.8	2.32 3.85	+ 65.9
1.07 1.02	−4.7	4.88 6.40	+ 31.1	3.24 4.07	+ 25.6
1.10 1.04	−5.5	3.00 5.35	+ 78.3	2.18 3.60	+ 65.1
1.11 1.00	−9.1	3.06 5.50	+ 79.7	2.14 4.03	+ 88.3
1.13 1.03	−8.8	2.55 6.50	+154.9	1.64 3.94	+140.2
1.08 0.99	−8.3	3.34 5.79	+ 73.2	2.37 4.47	+ 88.7
1.08 1.00	−7.4	3.35 5.92	+ 82.7	2.30 4.05	+ 81.3

The third and fourth columns in Table 1 show that after fertilization a slight decrease in the potassium content of the eggs usually occurred. As can be seen from the table, this decrease is not dependent on time.

Owing to the changes occurring subsequent to fertilization, a direct comparison of the potassium contents between unfertilized and fertilized eggs on basis of dry weight is, however, difficult.

If the eggs are more readily compressed by centrifuging after fertilization, as seems to be the case, the intercellular space would be less in this case. That is, there would be less salts coming from the surrounding sea-water in the dried material after fertilization than before. This should cause an apparent increase in the potassium percentage of the egg, since the artificial sea-water, with which the eggs were washed, did not contain potassium.

For this reason the authors also analysed the nitrogen and the phosphorus content of the eggs in the same samples, in order to calculate the amounts of potassium relative to the amount of phosphorus or nitrogen. This is a usual way to express the amount of other compounds in the sea-urchin egg (see, for instance, ÖMAN 1944) particularly since the total nitrogen and phosphorus content of the eggs are known not to be changed by fertilization.

Columns 5 to 8 show that, in general, the amounts of nitrogen and phosphorus also slightly decrease after fertilization. The potassium-nitrogen or potassium-phosphorus ratio therefore remains practically constant after fertilization.

In order to explain this tendency toward reduction in the potassium, nitrogen, and phosphorus content of the eggs, it was suggested by Mr S. LINDVALL, of this institution, that the content of other salts had increased in the egg after fertilization. The fertilization membrane is permeable to the ions in the surrounding sea-water. In this way new ions could be incorporated in the egg which must increase the salt content of the egg. Furthermore, no attempt was made in the experiments to release the eggs from their jelly coats. This can also contribute to the variations in the salt content of the eggs, because the ease with which the jelly coats are lost during washing can vary considerably from case to case.

Since the predominating cat- and anions in sea-water are sodium and chloride, the authors therefore determined the content of these ions. Columns 9–12, in Table 1, show that both these ions increase considerably after fertilization, and that the percentage of the increase is the same for both ions. From the table it can also be calculated that the amount of chloride, in the case of fertilized as well as unfertilized eggs, is practically equivalent to the amount of sodium. It is likely that almost all sodium chloride found in the unfertilized eggs originates from the intercellular water and not from the eggs themselves. As demonstrated by BIALASZEWICZ (1927), the unfertilized sea-urchin eggs contain but little sodium. This fact supports the opinion that there has been a free diffusion of the sea-water salts into the fertilization membrane and also explains the apparent slight decrease of some other ions in the egg.

Experiments with Strongylocentrotus droebachiensis

The eggs begin to divide about 5 hours after fertilization. There are no reports concerning the period during which acid formation takes place after fertilization. Samples were taken from 3/4 of an hour to 4 hours after fertilization. The results are recorded in Table 2.

As can be seen from columns 3 and 4, in Table 2, the potassium content of the eggs has again slightly decreased after fertilization. In this case also the decrease seems to be independent of the time.

The total nitrogen and phosphorus content of the eggs were determined, and again a slight decrease was noticed (columns 5 to 8 in Table 2). For this reason the authors determined sodium and chloride in this case too. These both ions increased considerably after fertilization, as in the case of *Psammechinus*. The increase in NaCl will thus again explain the slight decrease in the other ions examined in the egg.

Acknowledgments

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SUMMARY

A slight decrease in the potassium content of the sea-urchin egg takes place after fertilization. This decrease seems, however, to be only apparent. It was found that NaCl increased considerably after fertilization. This can be explained by the fact that the fertilization membrane is permeable to the ions in the surrounding sea-water. This increases the salt content in the egg, which, in turn, causes an apparent decrease in ions previously accumulated in the egg, when expressed in percentage of the dry weight.

From Wenner-Gren's Institute for Experimental Biology, University of Stockholm.

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Studies of siccatives

I. Kinetic investigations of the metal-catalysed autoxidation of methyl linoleate

By Börje Andersson

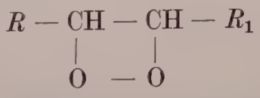
With 1 figure in the text

If linseed oil is brushed out into a thin layer and exposed to the air (oxygen), it will gradually assume a more or less solid consistency — the oil dries. In the drying process one can distinguish three main types of reaction, which in general interact, namely

1. *autoxidation*, during which the oil absorbs oxygen from the air,
2. *molecule enlarging* processes and, conditioned on same, *colloidchemical* processes,
3. *molecule diminishing* processes.

Even on the subject of the autoxidation there are many points of essential importance which still remain unclear, especially with regard to the metal-catalysed reaction, and we have only vague ideas about the fate of the autoxidation products in connection with the molecule enlarging and molecule diminishing processes.

Since the end of the 1800s there has in general been agreement that the *point of attack* for the autoxidation of linseed oil were to be looked for in the *unsaturated centers* of the linoleic and linolenic acids, and that the primary product is a *peroxide* (2, 21). Many investigators (e.g. 19, 20, 22, 36, 37, 38, 40) have accepted the theory introduced by Engler (21), Bach (2) and others, according to which the peroxide is formed through the addition of one oxygen molecule to the double bond with the formation of a *four-membered ring* containing the two oxygen atoms and the two carbon atoms of the double bond:



$$\begin{array}{ccccccc} R & - & \mathrm{CH} & - & \mathrm{CH} & - & R_1 \\ & & | & & | & & \\ & & \mathrm{O} & - & \mathrm{O} & & \end{array}$$

In support of this theory was presented inter alia the great tendency in general of unsaturated compounds to enter into addition reactions, and the observation that the unsaturation — measured by the iodine value — falls during the autoxidation at approximately the same rate as the peroxide content increases. It has been proved however that this observation can in part be traced to defects in the methods for determining the iodine value of compounds with conjugated double bonds (see p. 453). Besides, secondary processes must also in part be regarded as responsible for the diminution of the iodine value.

A new theory was presented about the middle of the 1930s which seems to furnish a better explanation of a number of later discovered phenomena. Thus, according to RIECHE (56), the primary product of autoxidation is a *hydroperoxide*, resulting from the entry of an oxygen molecule between the carbon and the hydrogen in a methylene group on the boundary of the double bond. Investigations by HOCK (45), CRIGEE (17), HELLBERG (43), TREIBS (63) and others of inter alia unsaturated hydrocarbons support this theory. In contradistinction to other investigators TREIBS (63) was of the opinion that the hydroperoxide group is linked to one of the double-bonded carbon atoms.

Owing to the relatively complex composition of linseed oil it was considered necessary toward the end of the 1930s to begin the study by using a more simply composed substance. For such substances have primarily been selected methyl and ethyl esters of oleic, linoleic and linolenic acids and simple hydrocarbons with isolated double bonds.

The hydroperoxide theory has been ever more securely confirmed since the beginning of the 1940s due to successful results in isolating almost pure hydroperoxide from autoxidized methyl oleate through molecular distillation (27), chromatography (27) or low-temperature fractional crystallization (62). It has been shown that the peroxide in autoxidized cyclohexene and other compounds can add on one mole of bromine per mole, and thus has maintained its unsaturation. It has further been found that it contains one mole of active hydrogen per mole. At reduction are formed saturated hydroxi-compounds corresponding to the hydroperoxide (e.g. 5, 6, 10, 23, 27, 32, 60). BOLLAND (10, 11), BERGSTRÖM (5, 6) and others have shown further that at autoxidation, say, of methyl and ethyl linoleate, ethyl linolenate etc., is refound the amount of oxygen absorbed during the primary process almost quantitatively in the form of peroxide. This does not however apply to all types of compounds with isolated double bonds such as, for example, many where the double bonds are separated by more than two single bonds (11, 24, 28, 29). At autoxidation of squalene, for instance, not quite half of the absorbed amount of oxygen is refound as peroxide.

While thus clearness may be said to exist with regard to the character of the peroxide formed at autoxidation of compounds with one or more isolated double bonds, this cannot be asserted about the reaction mechanism and the position of the peroxide group and double bonds in the autoxidation product.

The hydroperoxide formation at pure autoxidation appears at ordinary temperature and pressure to be *autocatalytical*. Good reasons implicate that *free radicals* appear as intermediate products, and that the reaction follows a *chain mechanism*. According to FARMER's original theory (25) the radical should arise through the removal of a hydrogen atom from an α-methylene group, and the hydroperoxide be formed through addition of the oxygen and re-combination of the hydrogen with the free valency. In consideration of the large amounts of energy that would be required for such a mechanism with regard to the large yield of peroxide which has been ascertained even at mild reaction conditions, FARMER has subsequently (31, 33, 34) modified his theory in so far that he assumes that there is primary addition of oxygen to the unsaturated center in a few molecules, e.g. through addition to the one end of the double bond. This primary product would then react by substitution with unaffected molecules under formation of free radicals, which in turn react further according to a chain mechanism. A similar theory, where the oxygen is primarily added on to the double bond, and then rearranged to a hydroperoxide, has been presented by Hilditch and others (42, 44). Bolland and others (3, 10, 11, 12) have subjected the chain mechanism to an incisive kinetic and thermochemical study, and have found valid support for Farmer's above described theory (see further p. 469).

The radicals which primarily arise at autoxidation of *singly unsaturated compounds* should, in consideration of the greater reactivity of the α-methylene groups, be of the following types.

$$R_1 - \underset{\bullet}{C}H - CH = CH - CH_2 - R_2 \text{ and } R_1 - CH_2 - CH = CH - \underset{\bullet}{C}H - R_2.$$

But these can rearrange to the mesomers

$$R_1 - CH = CH - \underset{\bullet}{C}H - CH_2 - R_2 \text{ resp. } R_1 - CH_2 - \underset{\bullet}{C}H - CH = CH - R_2$$

which would lead to the appearance of four isomeric hydroperoxides. This was postulated by Farmer-Koch-Sutton (30) in an investigation of polyolefines in 1943, and Swern, Knight, Scanlan, Ault (61) found in a study of the oxidation products of methyl oleate that a double bond shift must have taken place. Farmer—Sutton (32) found — through converting to the corresponding hydrogenation products — that, at autoxidation of 1: 2-dimethylcyclohexene-1 are formed both 1: 2-dimethylcyclohexene-1-hydroperoxide-3, and 1: 2-dimethylcyclohexene-2-hydroperoxide-1.

Ross-Gebhardt-Gerecht (57) demonstrated, inter alia through reduction and catalytic hydrogenation, that at autoxidation of methyl oleate are formed 8- and 11-hydroperoxide with the double bond between the carbon atoms 9 and 10, 9-hydroperoxide with the double bond between the carbon atoms 10 and 11, and 10-hydroperoxide with the double bond between the carbon atoms 8 and 9.

At *1: 4-unsaturated compounds* this mechanism lends itself to the formation of conjugated double bonds. From energetical reasons it can be concluded that the methylene group between the double bonds constitutes the principal seat of the primary reaction, inasmuch as the resonance energy connected with the release of a hydrogen atom from this is nearly twice as great as at other α-methylene groups. The radical primarily appearing, i.e.

$$R_1 - CH = CH - \underset{\bullet}{C}H - CH = CH - R_2$$

can rearrange into the mesomers

$$R_1 - \underset{\bullet}{C}H - CH = CH - CH = CH - R_2 \text{ and}$$
$$R_1 - CH = CH - CH = CH - \underset{\bullet}{C}H - R_2.$$

From energetical reasons it can be concluded that the conjugated modifications ought to predominate. Spectrographic investigations (UV) by Bergström (5, 6), Bolland-Koch (9), Lundberg (51, 52, 53), Holman (46, 47) and others have proved that conjugated dienes are formed at the autoxidation of linoleates but that only slightly more than 2/3 of the hydroperoxide is present in conjugated form. The expected conjugated hydroperoxides (9- and 13-hydroperoxide) have been revealed through converting to the corresponding saturated hydroxi-compounds, whereas it has not been possible to detect the 11-hydroperoxide. Bergström (6) does not deny the possibility that 11-hydroperoxide might have been at hand but maintains that, in such case, it must have been rearranged at the catalytic hydrogenation. Berg-

STRÖM (6) mentions as a more likely explanation that the very reactive conjugated hydroperoxide has reacted further with formation of nonconjugated compounds, thus resulting in lower values of conjugated peroxide. It is of course a wellknown fact that conjugated compounds are more rapidly oxidized than nonconjugated compounds owing to the 1: 4-addition of oxygen with resulting disappearance of the conjugation.

It would seem that at least during the earlier phase of the autoxidation only monohydroperoxides are formed and this is very probable due to energetical reasons (10, 11).

Even at autoxidation of *ethyl linolenate* it has been shown (26, 46, 47) through spectrographic analysis (UV) that conjugated compounds are formed, in this case both dienes and trienes. No absorption in the ultraviolet spectrum could on the other hand be traced in the case of *compounds with several double bonds, separated by more than two single bonds*, such as squalene etc. (30).

HILDITCH et al (1, 41) have studied the products obtained at autoxidation of methyl oleate at varying temperatures by subjecting these to oxidative decomposition. The results showed that, at autoxidation in temperatures round room temperature, the carbon atoms 8, 9, 10 and 11 are attacked whereas at temperatures round 100° C only the double-bonded carbon atoms 9 and 10 are attacked.

The considerable acceleration of the hydroperoxide formation by the circumstance of the α-methylene group being on both sides surrounded by double bonds has been shown by several investigators. STIRTON-TURER-RIEMENSCHNEIDER (59) found that the relative rates of oxygen absorption at 100° C of methyl oleate, methyl linoleate and methyl linolenate are 1, 10, 16 resp., and GUNSTONE-HILDITCH (41) found the corresponding rates at 20° C to be 1, 12 and 25 resp. These observations are also apt to support the hydroperoxide theory, as the rate of oxygen addition direct to the double bonds in the said compounds ought in reason to be of approximately the ratio 1: 2: 3. GUNSTONE-HILDITCH (42) have shown further that the presence of about 1 % methyl linoleate in a high degree accelerates the autoxidation of methyl oleate.

Comprehensive researches have thus been devoted to bring clearness in the reactions by which peroxides are formed at uncatalysed autoxidation of compounds with isolated double bonds. The investigations have also included catalysis by ultraviolet light (3, 4, 27, 30, 60 and others), peroxides (10, 11, 15), and enzymes (lipoxidase) (5, 7, 8, 48, 51), and the presence of inhibitors (13, 14, 51), in the course of which it has been shown that these factors do not alter the reaction mechanism.

In the practical use of linseed oil in paint and varnish manufacture however another type of catalysts is of greater interest, namely the so-called *siccatives*, or *driers*, which are composed of certain metallic salts, soluble in oil. The use of these is founded on the experience that if linseed oil is boiled together with white lead, red lead, or certain other lead compounds, the drying time is considerably shortened. For this reason lead became the solely used drier metal during several centuries. In the course of time it was learned to produce driers which are easily soluble in oils, and toward the end of last century lead and manganese compounds of linseed oil fatty acids and rosin acids were taken in use. From the beginning of the 1900s cobalt has been included in the family of paint driers. In recent years driers are also produced with saturated acids as anions, namely naphthenic acids and α-ethyl-caproic acid. Driers containing the last named acid are called octoates. Cobalt, manganese and lead remain to this day the predominating metals for production of paint driers, although certain other metals such as iron, zinc and calcium occur in special cases.

The processes through which these catalysts affect the drying of oils hitherto remain comparatively little investigated. This has in turn led to the presentation of more or less loosely founded hypotheses for the process in question. Among the many problems that have attracted great interest on this score is the question of which metals have a catalytic action and their mutual degree of efficiency. The relation between rate of reaction and metal concentration has also engaged the attention of several investigators. Unfortunately, most of the earlier observers adopted the drying time, determined according to dubious, more or less subjective methods as a measure of the reaction rate. Moreover, by studying the process up to the drying stage there is a risk that besides the autoxidation also molecule enlarging and diminishing reactions take place, something that will in a high degree make the interpretation of the results more difficult.

A very little better method for studying the autoxidation is the one of using so-called Weger curves. The oil is then spread in a thin layer on glass plates and exposed to the air. These are weighed at short intervals. The curves are obtained by plotting the relative weight increase of the oil films against the time. The weight-increase however is affected by a series of factors such as the escape of volatile oxidation products and absorption of moisture from the air. The restricted diffusion of oxygen into the film moreover contributes to making the results unsuitable to form the basis of theoretical discussions. Several investigators have nevertheless founded their theories on such observations.

It is hardly to be wondered at that different observers under these circumstances have arrived at conflicting results. Thus, e.g. EIBNER-PALLAUF (18) found the drying time of linseed oil, diminished by a constant, to be inversely proportional to the drier concentration. BOWLES (16) found at a study of cobalt-siccativated stand oil the drying time to be inversely proportional to the square root of the metal concentration, and LUND (50) arrived at the same conclusion at a study of cobalt and manganese driers in linseed oil. An investigation carried out by the MONTREAL PAINT & VARNISH PRODUCTION CLUB (55) went to show that proportionality should exist between the drying rate and drier concentration but that the proportionality constant would assume different values within different ranges of concentration!

The scientific investigations hitherto carried out concerning the correlation between reaction rate and drier concentration have mainly dealt with cobalt as the catalyst. It has for divers reasons proved difficult to study the process with manganese as the drier metal (see e.g. 50).

It has been stated on more or less doubtful premises that cobalt causes drying from the surface whereas manganese produces drying from the base. It is said that lead does not catalyse the primary autoxidation but merely the polymerization, and this metal is even regarded by many as not alone having any effect. These last statements however are disproved by the results presented in this paper.

Owing to the insufficient knowledge of the effect of the driers on the drying of oils, and the jumble of hypothesis this has brought out, it has been considered of interest to subject this question to a scientific study. In common with the study of the uncatalysed autoxidation it has been regarded as necessary to begin with a study of model substances, with a simpler composition than that of linseed oil.

The present investigation is confined to a study of the *autoxidation of methyl lin-*

oleate in the presence of the drier metals most commonly used, viz. cobalt, manganese and lead, each separately. The metals are introduced as salts of one and the same acid. The purpose of the investigation is to try to obtain enlightenment about the *relationship between the reaction rate and the concentration of catalyst* by means of a study of the reaction kinetics at the autoxidation of methyl linoleate when catalysed by metallic compounds, and to elucidate the *reaction mechanism at such autoxidation.*

Experimental

Methyl linoleate was prepared from poppy-seed oil, in the main according to McCutcheon et al (54). After saponification, the saturated acids and the main part of the oleic acid were separated through fractional crystallization from petroleum ether at —40° C. After brominating, the linoleic acid was isolated in the form of tetrabromstearic acid. After re-crystallization (melting point 115° C) it was debrominated — with simultaneous esterification — with zinc in methanol containing hydrogen chloride. The methyl linoleate was filtered, according to Bergström (6), in a petroleum ether solution through a column of activated alumina for the removal of peroxides and ev. free acid, and then distilled at reduced pressure. The colourless product was stored in sealed glass tubes, each containing about 0.2 g, under oxygen-free, dry nitrogen.

In the investigation was besides employed methyl linoleate purchased from the Hormel Foundation, Austin, Minn., U. S. A., which according to information had been prepared in a similar manner from cottonseed oil. This preparation was also kept in sealed tubes under nitrogen gas.

	Methyl linoleate	
	preparated by the author	from the Hormel Foundation
Iodine value accord. to Wijs[1]	172.0	172.4
Refractive index (n_D^{25})	1.4598	1.4595
Peroxide value (meqv/1000 g)	0	0

No differences were observed in the autoxidation experiments between the ester prepared by the author and the one purchased from the Hormel Foundation, and they have given quite identical results.

Butyl stearate was used to dilute the methyl linoleate (see p. 460). This compound was selected in consideration of it being a well defined, liquid non-volatile at 25° C, and for having good solubility in the reaction mixture used. Regarded as medium, it can be considered analogous to methyl linoleate, and as saturated compound it can be assumed to be indifferent to oxygen at the reaction temperature (25° C).

After distillation at reduced pressure the iodine value of the butyl stearate (accord. to Wijs) was < 0.1; $n_D^{25} = 1.4424$.

Driers: Cobalt, manganese and lead salts of α-ethyl-caproic acid were prepared through double replacement between water solutions of the sodium salt of α-ethyl-caproic acid and cobalt-II-sulphate, manganese-II-sulphate, or lead-II-nitrate. After extraction of the organic salt with carbon tetrachloride, washing with water and evaporation of the water at a higher temperature in vacuum, the driers were stored in the form of dilute solutions in carbon tetrachloride containing 0.002–2 mg of metal per ml. The solutions did not contain traceable quantities of sodium or of sulphate, or nitrate. Their metal content was determined after extraction with hot nitric acid through analysis in the usual way. The cobalt-salt solutions had a bluish-violet colour, the manganese-salt solutions were yellowish brown while the lead-salt solutions were colourless. α-ethyl-caproic acid was chosen as anion for being the only compound used in practice for this purpose with well defined composition. Both Dutch (18) and American (35) investigations seem to prove that naphthenates and octoates are approximately of equal value from a practical point of view. As the question of the effect of the anion will be dealt with in a later investigation, only salts of α-ethyl-caproic acid (octoates) were studied here.

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[1] Estimated value: 172.4.

Peroxide values were determined according to an iodometric method indicated by LEA (49), and slightly modified by SKELLON-WILLS (58). In a 300-ml Erlenmeyer flask was placed 20 ml of glacial acetic acid-chloroform (3: 2) which was freed from oxygen by means of through-bubbling with oxygen-free nitrogen gas during 5 minutes. 1.2 ml of potassium iodide solution, saturated at 18° C, was added, and the nitrogen gas through-bubbling was continued for 10 minutes, whereupon a sample of about 30 mg, weighed in a small glass tube, was added. After shaking, the mixture was quickly heated to boiling by immersing the flask in a boiling water bath. The flask was closed with a rubber stopper and the mixture was heated a further 2 minutes at 77° C, the content being then after cooling to room temperature, and addition of 50 ml of distilled water, titrated with 0.01-n $Na_2S_2O_3$ with starch as indicator. The peroxide value is stated in meqv. peroxidic oxygen per 1 000 g substance.

The autoxidation of methyl linoleate was studied by measuring the oxygen absorption at constant pressure and temperature in a specially designed set of apparatus, resembling the so-called "Warburg" equipment used for biochemical investigations. Fig. 1 shows the appearance of the apparatus. The reaction vessel A, a round-bottomed flask holding 100 ml, was immersed in a water thermostat at a temperature of 25°.18 ± 0°.01 C, and connected by means of capillary tubes (B) with the gas burette (C), holding 10 ml and graduated in 0.02 ml. D is a lever vessel by means of which the pressure in the reaction vessel is kept constantly the same as the atmospheric pressure outside. Dibutyl phthalate is used as blocking liquid. The drier — in a carbon tetrachloride solution — and ev. butyl stearate, was supplied into A in known quantities. The methyl linoleate was supplied direct from the storing tubes through the side tube E while evacuating through F (with stopcock G closed, H and I open), and the quantity was ascertained through weighing the storing tube before and after emptying. Before disconnecting the storing tube from E, to which it was attached with a rubber stopper during the admission of the ester, the apparatus was filled through F with oxygen-free nitrogen gas. The carbon tetrachloride was removed through evacuation during one hour while shaking. This proceeding ensured that the methyl linoleate did not come in contact with oxygen before the measurement was started. Pure and dry oxygen gas at a temperature of 25°.18 C was then let in through F.

The reaction vessel was shaken (with stopcock H closed, G and I open) during the experiments — except while the measuring results were read off — at the rate of 120 double strokes per min. and an amplitude of 6 cm. In order further to improve the agitation in the reaction vessel it contained six small glass pellets (diam. abt 6 mm).

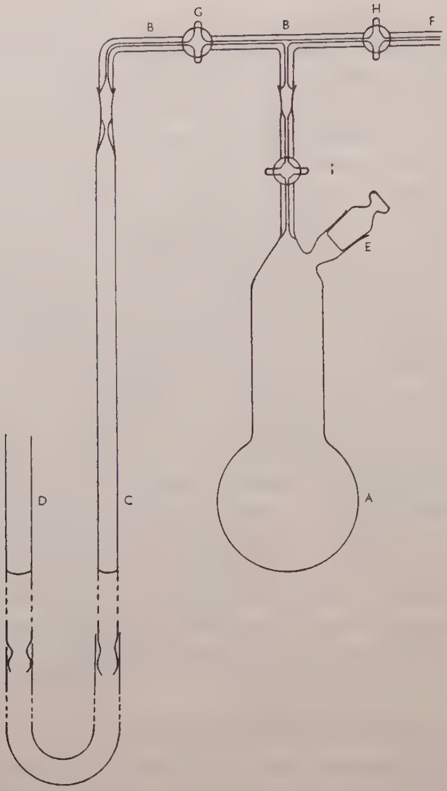
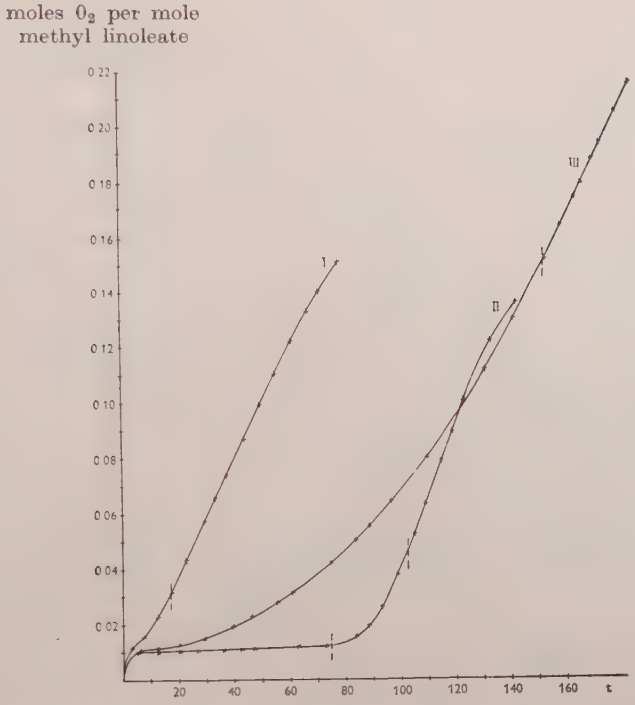


Fig. 1. Apparatus for determination of the oxygen absorption.

This proved necessary in order that the diffusion of oxygen into the reaction mixture should not affect the rate of reaction.

The oxygen absorption was read off at suitable intervals on the gas burette after the liquid level in D had been adjusted to the same height as in C. For affording facilities to make corrections to compensate alterations of the atmospheric pressure etc. was employed an exactly identical apparatus but with the reaction vessel empty, the gas burette of which was read off at the same times. From the readings was calculated the oxygen absorption in moles oxygen per mole methyl linoleate and the results plotted in a graph as a function of the reaction time. Some typical curves are reproduced in graph 1.

The *curves* show a short, steep rise to begin with, most likely correlated with the circumstance of the reaction mixture being then saturated with oxygen gas after the evacuation. Thereupon follows a range of varying length with autocatalytically increasing rate of absorption. In the presence of cobalt or manganese this range is of short duration, of lead considerably longer. At the autoxidation with manganese as catalyst the autocatalytic process is moreover preceded by an induction period of varying length during which there is none or extremely slight absorption of oxygen. The induction period has been found to be, in general, longer the higher the metal concentration. At diluted methyl linoleate the induction period is short, which is



Graph 1. Specimens of curves with the oxygen absorption of methyl linoleate as a function of the reaction time in the presence of

I. $2.88 \cdot 10^{-6}$ g-atoms Co/g
II. $3.09 \cdot 10^{-6}$ » Mn/g
III. $10.85 \cdot 10^{-6}$ » Pb/g

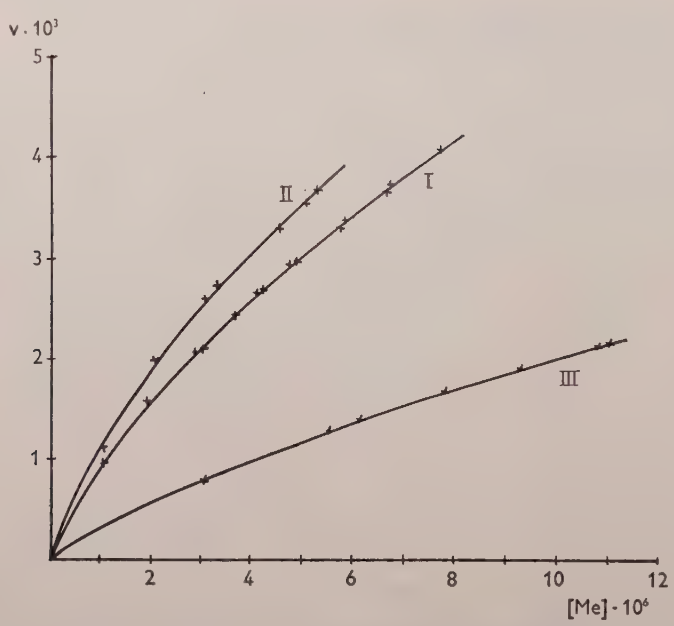
possibly connected with traces of oxidizing substances that may remain in the butyl stearate. Following the autocatalytic range is a period where the oxygen absorption rate is constant — at any rate within the attainable accuracy of measurement.

The general appearance of the curves makes it impossible to determine the rate of oxygen absorption with fair accuracy at the point of time 0. It was decided instead to determine the absorption rate within the rectilinear sections of the curves. These occur at approximately the same per cent of oxygen absorption in the presence of cobalt or manganese as catalyst, with lead at slightly higher per cent (see Table 2).

In order to ascertain whether the *agitation* and *quantity* of *ester* affect the reaction rate, some experiments were made at varying shaking velocities and quantities of ester. It was then found (see Table 1) that shaking at between 60 and 150 double strokes per minute, and quantities of ester between 0.1 and 0.3 g did not affect the results at the reaction rates in question.

The concentration of each of the three catalyst metals studied and the concentration of the methyl linoleate have been varied in the investigation. Furthermore there have been carried out a few tests at reduced oxygen pressure for deciding whether the reaction rate was appreciably dependent on such a factor.

The variation of the *metal concentration* had to be restricted to the values shown



Graph 2. Oxygen absorption rate of methyl linoleate at varying metal concentrations

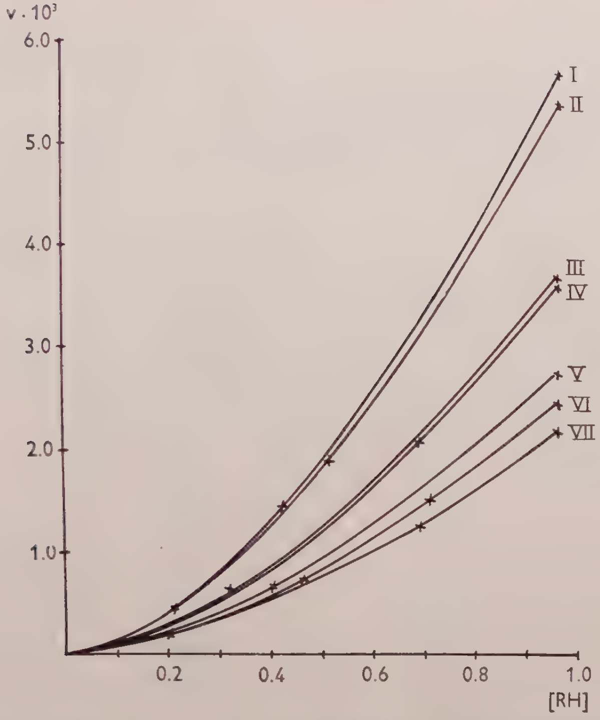
$[RH]_0 = 1; \quad p_{O_2} = 1.$

I. Cobalt. II. Manganese. III. Lead.

by Tables 4–6. This was done in order, on the one hand, to obtain so high a reaction rate that the experiments did not have to be extended over too long periods entailing risks of accompanying sources of faults, and, on the other hand, to ensure that the reaction rate should not attain so high values that the diffusion speed of the oxygen might play a part. In case of lead the upper limit of the metal concentration is set by the solubility of the drier. The concentrations studied have, as far as possible, been selected so as to correspond to the ones that occur in practice.

Variation of the *methyl linoleate concentration* has been effected by mixing the methyl linoleate with butyl stearate, in which case the last named ester was regarded as inactive to oxygen. In a corresponding manner the reduction of the *oxygen pressure* was effected by mixing the oxygen with nitrogen.

The results of the measurements are shown in Tables 4–10 and graphs 2–4. Seeing that the autoxidation rates at different concentrations of methyl linoleate in the presence of one and the same drier metal must be compared at the same oxidation level (i.e. the same peroxide and oxygen concentrations) in order for conclusions to be drawn from the figures (see p. 472 ff), these were studied for cobalt and manganese (graphs 3 and 4) at an oxygen absorption of $\frac{0.04}{[RH]_0}$ moles O_2 per mole of methyl linoleate ($[RH]_0$ = the methyl linoleate concentration at commencement of test in g/g reaction mixture) which according to Table 2 falls within the ranges of constant rate of reaction. The methyl linoleate concentrations corresponding to these



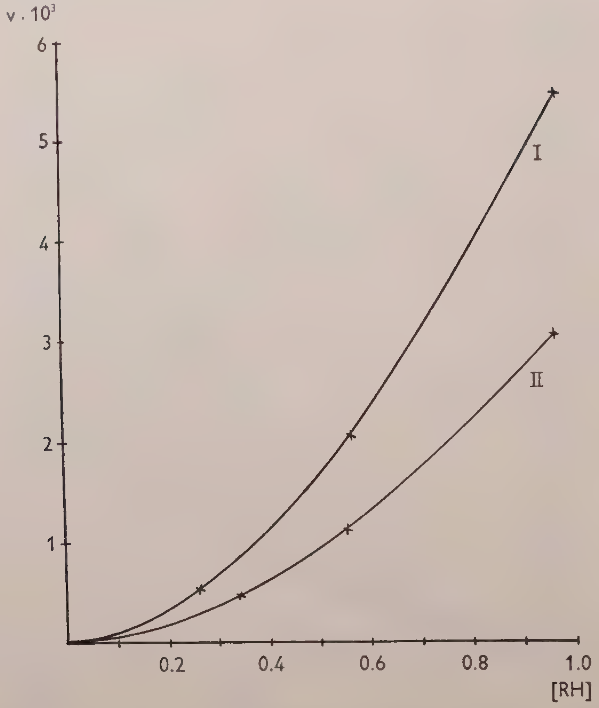
Graph 3. Oxygen absorption rate of methyl linoleate at varying methyl linoleate concentrations at the cobalt concentrations

I. $13.60 \cdot 10^{-6}$
II. $12.47 \cdot 10^{-6}$
III. $6.91 \cdot 10^{-6}$
IV. $6.66 \cdot 10^{-6}$
V. $4.44 \cdot 10^{-6}$
VI. $3.80 \cdot 10^{-6}$
VII. $3.24 \cdot 10^{-6}$

$p_{O_2} = 1$.

oxygen absorptions are $[RH]_0 - 0.04$. At the lead-catalysed autoxidation, it is not possible to find at varying methyl linoleate concentrations, any common peroxide concentration falling within the ranges of constant rate of reaction (see Table 2). The values in Table 9 at different concentrations of methyl linoleate in the presence of lead as catalyst can in consequence not be directly compared.

By means of determination of the *peroxide value* in the reaction mixture at the end of several of the experimental series, it has been found that the absorbed oxygen, as calculated from the absorption measurements, is refound almost quantitatively in the form of peroxide (Table 3). The reason why the peroxide was not refound in full quantity must no doubt in part be ascribed to the imperfection of the method of determination, but also to a minor decomposition of peroxide through secondary processes. At uncatalysed autoxidation BOLLAND (10) has earlier demonstrated that 90–100 % of the absorbed oxygen is recovered as peroxide.



Graph 4. Oxygen absorption rate of methyl linoleate at varying methyl linoleate concentrations at the manganese concentrations

I. $10.00 \cdot 10^{-6}$
II. $4.00 \cdot 10^{-6}$

$p_{O_2} = 1$.

The symbols used have reference to:

[Me] = metal concentration measured in g-atoms metal per g reaction mixture
[Co] = cobalt concentration measured in g-atoms cobalt per g reaction mixture
[Mn] = manganese concentration measured in g-atoms manganese per g reaction mixture
[Pb] = lead concentration measured in g-atoms lead per g reaction mixture
$[RH]_0$ = methyl linoleate concentration at commencement of test, measured in g methyl linoleate per g reaction mixture
[RH] = methyl linoleate concentration after the reaction time t measured in g methyl linoleate per g of reaction mixture
p_{O_2} = oxygen pressure measured in atm.
t = reaction time in minutes
v_0 = rate of oxygen absorption in moles O_2 per mole methyl linoleate (calculated on $[RH]_0$) and minute (= fraction of methyl linoleate oxidized per minute)
v = $[RH]_0 \cdot v_0$

The units of concentration may at first sight seem to be arbitrarily chosen, but that is not the case. [RH] is thus stated in g instead of in moles in order to obtain

$[RH] = 1$ for pure methyl linoleate which simplifies the mathematical treatment. The metal concentrations are on the other hand stated in g-atoms for eliminating the great difference in atomic weight between cobalt and manganese on the one hand, and lead on the other. The concentrations have been calculated for the sake of simplicity per g reaction mixture instead of per unit of volume, something that was regarded as permissible on account of the slight difference in spec. gravity of the different reaction mixtures.

Table 1

Rate of oxygen absorption of methyl linoleate at different shaking velocities and quantities of methyl linoleate.

$[RH]_0 = 1;\ p_{O_2} = 1.$

$[Co] \cdot 10^6$	Quantity of methyl linoleate (g)	Shaking velocity (double strokes per min.)	$v \cdot 10^3$
4.15	0.3010	60	2.66
4.12	0.2228	120	2.65
4.16	0.3092	120	2.65
4.21	0.1296	120	2.68
4.21	0.2506	120	2.67
4.20	0.1217	150	2.65
6.72	0.3020	60	3.50
6.67	0.0948	120	3.62
6.67	0.1346	120	3.63
6.75	0.2010	120	3.70
6.77	0.3015	120	3.70
6.74	0.1418	150	3.68

Table 2

Approximate limits of the ranges within which the metal-catalysed autoxidation rate is constant.

Kind of metal	$[RH]_0$	Lower limit	Upper limit
		of the oxygen absorption (moles O_2 per mole methyl linoleate) respecting the rectilinear sections of the primary curves	
Co	1	0.03	0.08—0.11
	0.75	0.04	0.12
	0.50	0.04	0.16
	0.25	0.04	0.17
Mn	1	0.03—0.04	0.09—0.10
	0.60	0.04	0.10
	0.40	0.05	0.14
	0.30	0.05	>0.16
Pb	1	0.17	0.23
	0.60	0.13	0.20
	0.30	0.13	0.20

Table 3

Peroxide content of methyl linoleate after autoxidation in the presence of cobalt, manganese or lead octoate.

$p_{O_2} = 1$.

Kind of metal	$[Me] \cdot 10^6$	$[RH]_0$	Experimentally determined oxygen absorption (a) (mmoles O_2 per 1000 g)	Peroxide value (b) (meqv. O_2 per 1000 g)	$\frac{b}{2\,a}$
Co	0.93	1	175	332	0.95
	2.88	1	580	1081	0.93
	4.12	1	445	839	0.94
	7.77	1	594	1117	0.94
	8.01	1	665	1279	0.96
	4.44	0.44	218	415	0.95
	12.47	0.55	295	549	0.93
Mn	1.03	1	306	581	0.95
	4.52	1	594	1120	0.94
	4.00	0.38	142	273	0.96
	10.00	0.30	122	227	0.93
Pb	5.56	1	841	1513	0.90
	7.81	1	912	1680	0.92
	10.45	0.32	260	473	0.91
	11.12	0.61	519	933	0.90

Table 4

Oxygen absorption rate of methyl linoleate at varying cobalt concentrations.

$[RH]_0 = 1$; $p_{O_2} = 1$.

$[Co] \cdot 10^6$	Quantity of methyl linoleate (g)	$v \cdot 10^3$
1.04	0.2103	0.95
1.90	0.2720	1.58
2.88	0.2306	2.05
3.02	0.1419	2.10
3.69	0.1750	2.43
4.12	0.2228	2.65
4.16	0.3692	2.65
4.21	0.1296	2.68
4.21	0.2506	2.67
4.74	0.1479	2.93
4.89	0.2153	2.95
5.73	0.1429	3.29
5.86	0.1399	3.35
6.67	0.1346	3.63
6.75	0.2010	3.70
7.77	0.1356	4.03

Table 5

Oxygen absorption rate of methyl linoleate at varying manganese concentrations. $[\mathrm{RH}]_0 = 1$; $p_{O_2} = 1$.

$[\mathrm{Mn}] \cdot 10^6$	Quantity of methyl linoleate (g)	$v \cdot 10^3$
1.03	0.2224	1.10
2.16	0.1902	2.00
3.09	0.2004	2.59
3.26	0.2028	2.68
4.52	0.2020	3.28
5.08	0.1968	3.54
5.32	0.1890	3.68

Table 6

Oxygen absorption rate of methyl linoleate at varying lead concentrations. $[\mathrm{RH}]_0 = 1$; $p_{O_2} = 1$.

$[\mathrm{Pb}] \cdot 10^6$	Quantity of methyl linoleate (g)	$v \cdot 10^3$
3.09	0.2151	0.79
5.56	0.2349	1.27
6.15	0.2013	1.38
7.81	0.2252	1.65
9.32	0.2218	1.88
10.85	0.2215	2.09
11.04	0.2041	2.11

Table 7

Oxygen absorption rate of methyl linoleate in the presence of cobalt at varying methyl linoleate concentrations.

$p_{O_2} = 1$.

$[\mathrm{Co}] \cdot 10^6$	Quantity of methyl linoleate (g)	Quantity of butyl stearate (g)	$[\mathrm{RH}]_0$	$v_0 \cdot 10^3$	$v \cdot 10^3$
3.24	0.2139	0.0774	0.73	1.73	1.26
3.80	0.1961	0.6237	0.24	0.78	0.19
3.82	0.2045	0.2034	0.50	1.47	0.74
3.88	0.2014	0.0661	0.75	2.04	1.53
4.44	0.1196	0.1536	0.44	1.55	0.68
6.91	0.1897	0.2790	0.40	1.85	0.74
6.66	0.1548	0.0568	0.73	2.85	2.08
12.47	0.2911	0.2410	0.55	3.45	1.90
13.60	0.1762	0.5442	0.25	1.77	0.44
13.76	0.1940	0.2314	0.46	3.22	1.48

Table 8

Oxygen absorption rate of methyl linoleate in the presence of manganese at varying methyl linoleate concentrations.

$p_{O_2} = 1.$

$[Mn] \cdot 10^6$	Quantity of methyl linoleate (g)	Quantity of butyl stearate (g)	$[RH]_0$	$v_0 \cdot 10^3$	$v \cdot 10^3$
4.00	0.1991	0.3195	0.38	1.23	0.47
3.96	0.2340	0.1645	0.59	1.88	1.11
10.00	0.1852	0.4306	0.30	1.79	0.54
9.97	0.1930	0.1271	0.60	3.40	2.04

Table 9

Oxygen absorption rate of methyl linoleate in the presence of lead at varying methyl linoleate concentrations.

$p_{O_2} = 1.$

$[Pb] . 10^6$	Quantity of methyl linoleate (g)	Quantity of butyl stearate (g)	$[RH]_0$	$v_0 \cdot 10^3$	$v \cdot 10^3$
5.81	0.2100	0.4667	0.31	0.74	0.23
5.83	0.1968	0.1119	0.64	1.09	0.70
10.45	0.2439	0.5228	0.32	1.11	0.36
10.44	0.2246	0.1560	0.59	1.59	0.94
11.08	0.2203	0.5149	0.30	1.12	0.34
11.12	0.2038	0.1301	0.61	1.66	1.01

Table 10

Oxygen absorption rate of methyl linoleate in the presence of cobalt, manganese or lead octoate at reduced oxygen pressure.

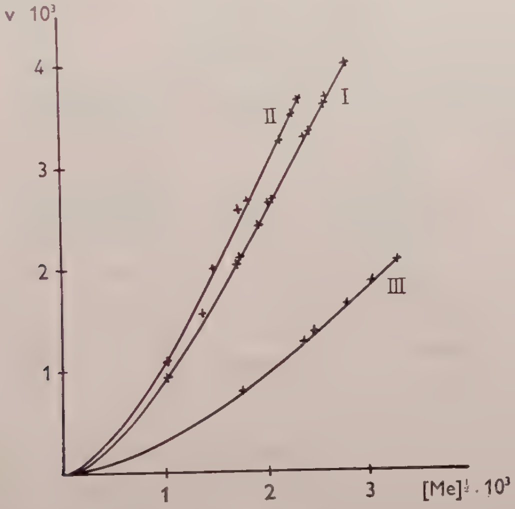
Kind of metal	$[Me] \cdot 10^6$	p_{O_2}	Quantity of methyl linoleate (g)	$v \cdot 10^3$
Co	3.69	$\frac{1}{2}$	0.1933	2.05
Co	5.57	$\frac{1}{2}$	0.1400	2.85
Mn	3.24	$\frac{1}{2}$	0.2251	2.54
Pb	11.13	$\frac{1}{2}$	0.2210	1.65

Mathematical analysis

Graph 2 shows directly that the reaction rate of metal-catalysed autoxidation of *undiluted methyl linoleate* cannot be proportional to the metal concentration. Moreover, a mathematical analysis of the present values shows that neither does proportionality between the reaction rate and the square root of the metal concentration entirely agree with the experimental data (see also graph 5). The analysis points to a parabolic function

$$v^2 + a \cdot v = \beta \cdot [\mathrm{Me}] \qquad \text{(I)}$$

where α and β are two factors which are independent of the metal concentration. This applies to both *cobalt* and *manganese*, as well as *lead*.



Graph 5. Oxygen absorption rate of methyl linoleate as a function of $\sqrt{[\mathrm{Me}]}$ at $[\mathrm{RH}]_0 = 1$; $p_{O_2} = 1$.

I. Cobalt. II. Manganese. III. Lead.

An examination of the results shows further that the factors α and β inter alia depend on the *methyl linoleate concentration* (Table 11).

A mathematical analysis of the values in Table 11 a and b shows that the following expressions apply for the *cobalt* or *manganese* catalysed autoxidation at *constant* $[O_2]$ and one and the same $[ROOH]$ (= 0.04), viz.

$$a = \frac{a_1 \cdot [\mathrm{RH}]^2 \cdot ([\mathrm{RH}] + a_2)}{[\mathrm{RH}] + \beta_2},$$

$$\beta = \frac{\beta_1 \cdot [\mathrm{RH}]^4}{[\mathrm{RH}] + \beta_2},$$

i.e.

$$v^2 + \frac{a_1 \cdot [\mathrm{RH}]^2 \cdot ([\mathrm{RH}] + a_2)}{[\mathrm{RH}] + \beta_2} \cdot v = \frac{\beta_1 \cdot [\mathrm{RH}]^4}{[\mathrm{RH}] + \beta_2} \cdot [\mathrm{Me}]. \tag{II}$$

α_1, α_2, β_1, and β_2 are independent of the methyl linoleate concentration. Whether the same expressions also apply to autoxidation with *lead* as catalyst cannot be decided from the available experimental material (see p. 460), but nothing contradicts this assumption.

The values of a_1, a_2, β_1 and β_2 calculated from the figures obtained, are presented in Table 12.

From Table 13 is seen the agreement between the experimentally obtained values and the values of v for a few metal and methyl linoleate concentrations as calculated on the basis of Table 12.

Table 11

Values of the factors a and β at varying concentrations of methyl linoleate.

a. In the presence of cobalt.

$[\mathrm{RH}]_0$	$[\mathrm{RH}]$[1]	$a \cdot 10^3$	β
1.00	0.96	3.41	3.86
0.75	0.71	2.04	1.41
0.50	0.46	0.97	0.33
0.25	0.21	0.25	0.02

b. In the presence of manganese.

$[\mathrm{RH}]_0$	$[\mathrm{RH}]$[1]	$a \cdot 10^3$	β
1.00	0.96	2.84	4.46
0.75	0.71	1.73	1.54
0.50	0.46	0.83	0.33
0.25	0.21	0.21	0.018

c. In the presence of lead.

$[\mathrm{RH}]_0$	$[\mathrm{RH}]$[1]	$\alpha \cdot 10^3$	β
1.00	0.80	2.95	0.97
0.60	0.50	1.18	0.20
0.30	0.25	0.50	0.03

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[1] See page 460.

Table 12

Values of α_1, α_2, β_1 and β_2 at autoxidation of methyl linoleate with cobalt or manganese as catalyst

Metal	α_1	α_2	β_1	β_2
Co	$2.2 \cdot 10^{-3}$	1.3	6.1	0.4
Mn	$0.9 \cdot 10^{-3}$	4.9	9.2	0.8

Table 13

Kind of metal	$[Me] \cdot 10^6$	$[RH]_0$	$v \cdot 10^3$	
			Experimentally obtained	Calculated
Co	1.04	1	0.95	0.92
	4.21	1	2.68	2.66
	5.86	1	3.35	3.33
	7.77	1	4.03	4.01
	3.80	0.24	0.19	0.19
	3.82	0.50	0.74	0.72
	3.88	0.75	1.53	1.51
Mn	1.03	1	1.10	1.16
	3.09	1	2.59	2.57
	5.08	1	3.54	3.56
	4.00	0.38	0.47	0.46
	3.96	0.59	1.11	1.11

Discussion

The experiments have plainly shown that each of the metals *cobalt*, *manganese* and *lead* will *catalyse* the *primary oxygen absorption* of methyl linoleate though the action of lead is considerably weaker than of the two other metals. Manganese has proved somewhat more effective than cobalt but this is offset by a longer induction period. The relationship found between reaction rate and metal concentration seems to indicate that there is here not a question of an ordinary, simple chemical reaction.

BOLLAND-GEE (10, 11), as already mentioned, found that *uncatalysed* autoxidation of ethyl linoleate proceeds mainly by the following chain mechanism:

Initiation 1. $2\,ROOH \rightarrow R$ or RO_2
Propagation 2. $R + O_2 \rightarrow RO_2$
3. $RO_2 + RH \rightarrow ROOH + R$
Termination 4. $R + R \rightarrow$ inactive product
5. $R + RO_2 \rightarrow$ inactive product
6. $RO_2 + RO_2 \rightarrow$ inactive product.

R here refers to a free radical which is formally derived through the removal of one hydrogen atom from an α-methylene group in the linoleate. RO_2 represents the the same radical upon the addition of one molecule of oxygen, but still retaining the radical nature. ROOH, finally, refers to the linoleate hydroperoxide.

At oxygen pressures above 100 mm Hg, BOLLAND-GEE (10, 11) found that the termination reaction 6 is so predominant that the other two (i. e. 4 and 5), may be neglected. From this they derived the oxygen absorption rate

$$v = k \cdot [\mathrm{RH}] \cdot [\mathrm{ROOH}].$$

At autoxidation *catalysed by dibenzoylperoxide* (shortened below to Dib) they found (10, 11, 15) a resembling mechanism, with the only difference that the initiation reaction was replaced by Dib→R or RO_2 whereby v obtained the form $k \cdot [\mathrm{RH}] \cdot [\mathrm{Dib}]^{1/2}$.

As the result of thorough investigations they found this mechanism in good agreement with chemical, kinetic and thermochemical data.

Strong support was found by LUND (50) from his investigation of *manganese* and *cobalt driers in linseed oil* for the theory launched long since, that the drying effect is connected with the ability of valency alteration of the drier metals. He showed that Mn^{+2} and Co^{+2} lack catalysing effect which seems to be attached to higher states of valency of the metals in question. Lund showed further that if an originally peroxide-free siccativated linseed oil is exposed to the action of the air in a thin layer there is an incipient induction period with insignificant absorption of oxygen, during which the metal is oxidized to higher states of valency under the influence of peroxides formed in the oil. It was found that a "minimum oxidation level" was necessary for any appreciable quantities of metal being present in the higher-valent state. After this level had been attained, the oxygen absorption, catalysed by the metals, proceeds rapidly. Lund showed that manganese demands a considerably higher oxidation level than cobalt, which results in a long induction period for manganese-siccativated linseed oil, whereas the induction period in case of cobalt-siccativated oil is of short duration. This induction period for manganese as drier, as already mentioned, has also proved to be at hand at autoxidation of methyl linoleate. By means of supplying suitable oxidizing agents, Lund showed further that the oxidation level could be raised, so that also manganese-siccativated oil dried without any induction period. Moreover, through the addition of suitable reducing agents it was possible to get cobalt-siccativated oil to dry with a considerable induction period. On the basis of these observations Lund formulated the proposition that the drier effect is determined by 1) the proportion of the metal present in the higher-valent state and 2) the reducibility from the higher-valent state. Lund presented a chain mechanism for the reaction, with a linseed oil-metal complex as chain carrier, without however giving any direct proof. Such oil-metal complexes have been discussed by several investigators in this sphere of research. GEORGE et al (39) have besides, in studies of metal-catalysed autoxidation of tetralin found signs which point to the occurrence of similar complexes even there.

In consideration for one thing, to these earlier findings, and to the results of this investigation, it seems fairly reasonable to assume a chain mechanism for the *metal-catalysed autoxidation of methyl linoleate*, resembling the one set up by BOLLAND et al for the uncatalysed reaction. The chain carriers are probably addition products of radical character between the metal and the unsaturated ester. Having regard to the initially autocatalytically increasing reaction rate, there is probably included oxidation of the metal with the aid of peroxide as a link in the initiation reaction. The following reaction scheme complies with the above demands and has proved in good agreement with the experimental results (see below).

			Reaction velocity constant
Initiation	1.	$Me^* + ROOH \rightarrow MeO_2^* +$ inactive prod.	k_1
	2.	$MeO_2^* + RH \rightarrow RMeO_2 +$ » »	k_2
Propagation	3.	$RMeO_2 + RH \rightarrow RMe + ROOH$	k_3
	4.	$RMe + O_2 \rightarrow RMeO_2$	k_4
Termination	5.	$RMeO_2 + RMeO_2 \rightarrow 2\ Me +$ inactive prod.	k_5
	6.	$RMeO_2 + RMe \rightarrow 2\ Me +$ » »	k_6
	7.	$RMe + RMe \rightarrow 2\ Me +$ » »	k_7

RMe and $RMeO_2$ represent two free radicals, corresponding to R and RO_2, (see p. 469) of the uncatalysed autoxidation but in addition containing metal, in this case linked to α-ethyl-caproic acid. Me* and MeO_2* refer to addition products between octoate of the metal in the lower and higher oxidation state, resp., and methyl linoleate. It is only through this addition to methyl linoleate that the metal becomes active. The addition product Me* can be regarded as having been formed already during the evacuation period that precedes the autoxidation (see p. 457), during which equilibrium is established between free metal octoate (designated below Me_f) and metal octoate added to methyl linoleate.

$$Me_f + RH \rightleftarrows Me^*.$$

It seems fairly well founded to make the assumptions that only a small fraction of the octoate undergoes such addition, and that the concentration of metal added to the methyl linoleate, based on the law of mass action, can be estimated as

$$[Me^*] = K \cdot [Me] \cdot [RH]$$

where K represents the equilibrium constant and [Me] the total metal concentration (i.e. the content of metal supplied to the reaction mixture in the form of drier). The total active metal concentration ($[Me^*] + [MeO_2^*] + [RMe] + [RMeO_2]$) can be assumed constant during the autoxidation. The decreased reaction to the left in the above reaction formula, due to decrease of [Me*] through its conversion into other active products (MeO_2*, RMe and $RMeO_2$) is compensated by the termination reactions causing active metal to be desactivated.

At the comparatively high oxygen pressure we are dealing with, the chain termination reactions 6 and 7 can be neglected by the side of 5. The above reaction scheme will then, at stationary state, give rise to the following equations:

$$\frac{d[RMe]}{dt} = 0 = k_3 \cdot [RMeO_2] \cdot [RH] - k_4 \cdot [RMe] \cdot [O_2]$$

$$\frac{d[RMeO_2]}{dt} = 0 = k_2 \cdot [MeO_2] \cdot [RH] - k_3 \cdot [RMeO_2] \cdot [RH] + k_4 \cdot [RMe] \cdot [O_2] - k_5 \cdot [RMeO_2]^2$$

$$\frac{d\,[\mathrm{MeO_2}^*]}{dt} = 0 = k_1 \cdot [\mathrm{Me}^*] \cdot [\mathrm{ROOH}] - k_2 \cdot [\mathrm{MeO_2}^*] \cdot [\mathrm{RH}]$$

$$v = k_4 \cdot [\mathrm{RMe}] \cdot [\mathrm{O_2}].$$

From this we obtain:

$$[\mathrm{RMe}] = \frac{v}{k_4 \cdot [\mathrm{O_2}]}$$

$$[\mathrm{RMeO_2}] = \frac{v}{k_3 \cdot [\mathrm{RH}]}$$

$$[\mathrm{MeO_2}^*] = \frac{k_5 \cdot v^2}{k_2\, k_3{}^2 \cdot [\mathrm{RH}]^3}$$

$$[\mathrm{Me}^*] = \frac{k_5 \cdot v^2}{k_1\, k_3{}^2 \cdot [\mathrm{RH}]^2 \cdot [\mathrm{ROOH}]}$$

$$\because \sum [\mathrm{Me}]_{\mathrm{active}} = [\mathrm{Me}^*] + [\mathrm{MeO_2}^*] + [\mathrm{RMe}] + [\mathrm{RMeO_2}] =$$
$$= \frac{k_5 \cdot v^2 \cdot (k_2 \cdot [\mathrm{RH}] + k_1 \cdot [\mathrm{ROOH}])}{k_1\, k_2\, k_3{}^2 \cdot [\mathrm{RH}]^3 \cdot [\mathrm{ROOH}]} + \frac{v \cdot (k_3 \cdot [\mathrm{RH}] + k_4 \cdot [\mathrm{O_2}])}{k_3\, k_4 \cdot [\mathrm{RH}] \cdot [\mathrm{O_2}]} =$$
$$= \mathrm{K} \cdot [\mathrm{RH}] \cdot [\mathrm{Me}] \text{ (see above).}$$

$$\because v^2 + \frac{k_1\, k_3{}^2}{k_4\, k_5} \cdot \frac{[\mathrm{ROOH}]}{[\mathrm{O_2}]} \cdot \frac{[\mathrm{RH}]^2 \cdot \left([\mathrm{RH}] + \frac{k_4}{k_3} \cdot [\mathrm{O_2}]\right)}{[\mathrm{RH}] + \frac{k_1}{k_2} \cdot [\mathrm{ROOH}]} \cdot v =$$
$$= \frac{\mathrm{K}\, k_1\, k_3{}^2}{k_5} \cdot [\mathrm{ROOH}] \cdot \frac{[\mathrm{RH}]^4}{\frac{k_1}{k_2} \cdot [\mathrm{ROOH}] \cdot [\mathrm{RH}]} \cdot [\mathrm{Me}]. \qquad \text{(III)}$$

If $[RH]$, $[ROOH]$ and $[O_2]$ be regarded as *constants* and $[Me]$ as a *variable*, this expression is simplified to

$$v^2 + \alpha' \cdot v = \beta' \cdot [\mathrm{Me}] \qquad \text{(IV)}$$

where in α' and β' are combined resp. constant terms.

If $[ROOH]$ and $[O_2]$ be regarded as *constants* and $[RH]$ and $[Me]$ as *variables*, the expression is transformed to

$$v^2 + \frac{\alpha_1' \cdot [\mathrm{RH}]^2 \cdot ([\mathrm{RH}] + \alpha_2')}{[\mathrm{RH}] + \beta_2'} \cdot v = \frac{\beta_1' \cdot [\mathrm{RH}]^4 \cdot [\mathrm{Me}]}{[\mathrm{RH}] + \beta_2'}. \qquad \text{(V)}$$

where

$$\alpha_1' = \frac{k_1\, k_3{}^2 \cdot [\mathrm{ROOH}]}{k_4\, k_5 \cdot [\mathrm{O_2}]}, \qquad \alpha_2' = \frac{k_4 \cdot [\mathrm{O_2}]}{k_3}$$
$$\beta_1' = \frac{\mathrm{K}\, k_1\, k_3{}^2}{k_5} \cdot [\mathrm{ROOH}], \qquad \beta_2' = \frac{k_1 \cdot [\mathrm{ROOH}]}{k_2}. \qquad \text{(VI)}$$

The experimentally found equation (I) and the theoretically derived equation (IV), which are conditioned by the same requisites, i.e. constant (RH), [ROOH] and $[O_2]$, varying [Me], agree wholly in form when α is identified with α' and β with β'. The same is valid for the equations (II) and (V), which are conditioned by constant [ROOH] and $[O_2]$, variable [RH] and [Me], when α_1, α_2, β_1 and β_2 are identified with α'_1, α'_2, β'_1 resp. β'_2.

Additional support for the correctness of the foregoing chain mechanism is afforded through a calculation of the oxygen absorption rates at constant [Me], $[O_2]$ and $[RH]_0$ and varying [ROOH]; i.e. *reaction rates* in one and the same experiment *at different reaction times*, on the basis of (II) and values in Table 12 while keeping in mind that $\alpha_1 = \alpha'_1$, $\alpha_2 = \alpha'_2$, $\beta_1 = \beta'_1$, $\beta_2 = \beta'_2$. It will then be found that the results agree with the ones found experimentally. In other terms, there is an initial short period during which the rate increases with the peroxide concentration, and subsequently a range with almost constant rate independently of the peroxide concentration. In Table 14 are given some examples of the results of these calculations for a few metal concentrations. The small differences brought out between the experimental and the calculated values, in particular at higher metal concentrations, is explained by a certain decomposition of peroxide which has not been taken into account at the calculations. Calculation of the effect of a *reduction* of the *oxygen pressure* from 1 to 1/2 atm on the reaction rate — on the basis of the same equations as above — also proves to be in agreement with the experimental results in Table 10.

A study of the values of the constants α_1, α_2, β_1 and β_2 identified with α'_1, α'_2, β'_1 resp. β'_2 furnishes certain further information. From (VI) is thus obtained

$$k_1 = k_2 \cdot \beta_2 \cdot \frac{1}{[\mathrm{ROOH}]}, \qquad \text{(VII)}$$

$$k_4 = k_3 \cdot \alpha_2 \cdot \frac{1}{[\mathrm{O_2}]} = \frac{\beta_1}{\mathrm{K}\,\alpha_1} \cdot \frac{1}{[\mathrm{O_2}]}. \qquad \text{(VIII)}$$

The constants K, k_2, k_3 and k_5 can no doubt be assumed to be comparatively independent of which metal is used as catalyst. From (VII) and (VIII) is seen that initiation reaction 1 (p. 471) possesses twice as high reaction velocity constant in the presence of manganese as of cobalt, and reaction 4 approx. 3 1/2 times as high. This proves that with cobalt and manganese as drier the oxidability of the metal is at any rate one of the factors which determines their comparative efficiency. Whether this is of general applicability will not be decided here even if from practical experience this seems rather probable.

As concerns, finally, the observed induction period with manganese as drier, no explanation has been found. It is just possible that this feature is correlated with the reactions which take place at the initial formation of peroxide.

Lund (50) presents in his work the opinion, also favoured by many other investigators, that the metal concentration has an optimal value, and that higher concentrations may even have a retarding effect. The truth is probably not to be found in an actual optimum but is very likely connected partly with the solubility of the drier and partly with the rate of oxygen diffusion into the oil. This last factor would seem to have been overlooked in earlier investigations into this matter. The fact that it constitutes an essential magnitude is proved inter alia by the necessity in the course of the author's investigation to take rigorous measures to attain so high a diffusion

Table 14

Oxygen absorption by methyl linoleate at different reaction times, calculated on the basis of equations (II), (V) and (VI) and Table 12.

a. In the presence of cobalt octoate.

[ROOH]	[RH]	$v \cdot 10^3$ at		
		[Co] = $2 \cdot 10^{-6}$	[Co] = $4 \cdot 10^{-6}$	[Co] = $8 \cdot 10^{-6}$
0.01	0.99	1.16	1.81	2.75
0.02	0.98	1.37	2.23	3.43
0.03	0.97	1.49	2.44	3.82
0.04	0.96	1.56	2.58	4.07
0.05	0.95	1.57	2.62	4.18
0.06	0.94	1.59	2.67	4.29
0.08	0.92	1.59	2.70	4.38
0.10	0.90	1.57	2.69	4.38
0.12	0.88	1.54	2.64	4.32
0.15	0.85	1.47	2.51	4.20
0.20	0.80	1.35	2.35	3.90

b. In the presence of manganese octoate.

[ROOH]	[RH]	$v \cdot 10^3$ at	
		[Mn] = $2 \cdot 10^{-6}$	[Mn] = $5 \cdot 10^{-6}$
0.01	0.99	1.36	2.43
0.02	0.98	1.71	3.09
0.03	0.97	1.84	3.37
0.04	0.96	1.90	3.52
0.05	0.95	1.91	3.57
0.06	0.94	1.93	3.63
0.08	0.92	1.92	3.58
0.10	0.90	1.77	3.58
0.12	0.88	1.73	3.50
0.15	0.85	1.72	3.34
0.20	0.80	1.57	3.04

rate that this did not affect the reaction. If so large a quantity of drier is added that the reaction rate is wholly determined by the oxygen diffusion, there will naturally be no shortening of the drying time by adding more metal. Nothing in the author's investigation indicates that a real optimal metal concentration can be fixed upon.

In judging the practical consequences of the author's investigation it should be kept in mind that only the reactions that take place during the primary autoxidation have been subjected to study. The experiments in consequence cannot directly be made the basis for judging the influence of the driers on the drying time of the oil, seeing that rates of the secondary processes play a part. It is impossible, on the basis of these experiments, to determine how the secondary processes are affected by the driers. It must further be remembered that the reaction rate during the autoxidation certainly increases with the metal concentration according to a parabolic relationship, but that the induction period which has proved to be of varying length also contributes to determine the length of the drying time. It has thus been shown e.g. that manganese accelerates the autoxidation in a slightly higher degree than cobalt at the same metal concentration, but this is offset by the much longer induction period for manganese than for cobalt. This carries with it that the degree of autoxidation after a certain length of time usually is higher for cobalt than for manganese of the same concentration.

In investigations planned will be studied inter alia the action of more metals in combination, and of other metals than cobalt, manganese and lead, on the autoxidation of methyl linoleate.

The present paper forms part of the fundamental research undertaken by the Central Research Laboratory of the Swedish Paint and Varnish Industry.

The author is greatly indebted to Professor PAUL NYLÉN who proposed the subject for the present investigation, for the benefit derived from many valuable discussions and the interest taken by him in the prosecution of the task.

SUMMARY

Through kinetic investigations it has been shown that the relationship between the rate (v) of the metal-catalysed autoxidation of methyl linoleate and the metal concentration ([Me]) is given by the equation $v^2 + \alpha \cdot v = \beta \cdot [\mathrm{Me}]$. The investigation which has comprised cobalt, manganese and lead catalysts has proved that all three of the metals catalyse the autoxidation but that the action of lead is considerably lower than of the other two metals. The autoxidation rate depends in a high degree on the methyl linoleate concentration but is only to a slight extent affected by a lowering of the oxygen pressure from 1 to 1/2 atm. The results point to a chain mechanism resembling the one which determines the uncatalysed autoxidation but with chain carriers of radical character consisting of metal added to radicals of similar type as at the uncatalysed reaction.

Central Research Laboratory of the Swedish Paint and Varnish Industry, Stockholm.

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On filtration through a sorbent layer

IV. The Ψ condition, a simple approach to the theory of sorption columns

By LARS GUNNAR SILLÉN

With 14 figures in the text

The previous papers in this series (I–III) have been concerned with the "fronts" or "leaking curves" obtained when a solution is passed through a sorbent layer. This paper will deal with the limiting case, where *equilibrium* between sorbent and solution is obtained at every point of the sorbent layer. In many types of experiments equilibrium is approximately obtained and, as will be discussed in the next paper (Part V), the processes at equilibrium provide a good basis for understanding the cases of non-equilibrium filtration.

This paper will deal with two different cases: in the *general case*, no assumption is made regarding the influence of one sorbate on the other, whereas for *ion exchange* it is assumed that a certain amount of an ion can only be sorbed by displacing an equivalent amount of other ions. Under rather general conditions (3 a, 3 b) the concentrations of all substances will be functions of the single variable $\Psi = \frac{V}{x}$ (2); in such cases the shape of the sorption fronts can be calculated by means of the very simple formulae (4) or (5). For an ion exchanger it is preferable to use instead a different variable ψ (15) which is proportional to Ψ. In this case the conditions will be (16 a, b), and the formula for the front will be (17) or (18). These formulae will be applied to a few important processes, both in the general case and in ion exchange.

Some of the results obtained here coincide with those of previous authors on the theory of chromatography, especially DE VAULT (1943) and WEISS (1943).

When this paper was first worked out, I was unaware of the many important contributions to the theory of chromatography, that were given in the FARADAY discussion at Reading, on Sept. 22nd—24th, 1949. Professor ARNE TISELIUS, who was kind enough to read my manuscript, has shown me the proofs of these papers and pointed out to me that many of my results had already been derived by GLUECKAUF (1949) and by OFFORD and WEISS (1949). The latter have in special cases used an approach which is essentially equivalent to the "Ψ condition". However, the simplification that can be obtained by using it consistently does not seem to have been generally taken advantage of. For instance, GLUECKAUF's (1949) equations (12 a, b) might have been written down immediately as consequences of the Ψ condition.

Since the Ψ condition seems to give a uniform, quick, and simple way of treating various processes in sorption columns, it was nevertheless thought that it might be worth while demonstrating its application to a number of cases.

The Ψ condition has also proved valuable in the treatment of non-equilibrium conditions, as will be shown in the next paper (Part V). In papers to follow, EKEDAHL and HÖGFELDT will report on applications to leaking curves and equilibria of ion exchangers.

Coordinates

A solution is led into an infinitely long tube, which is uniformly filled with one and the same sorbent. For describing the system we need two coordinates: x, which is fixed relative to the sorbent, and V, which is fixed relative to the solvent (Fig. 1 a). The distance from that end ($x = 0$), where the solution enters the sorbent, is x cm. A certain layer of the solution is characterized by V ml, the volume between that layer and the first layer ($V = 0$) that has entered the sorbent. At a given moment, the value of V for different x values in the column is not the same, but is given by a sloping line like those in fig. 1 b. In the following, the conditions in the column will often be represented by diagrams with V and x as coordinates; what is observed in actual experiments is mostly the solutions obtained at a given x (vertical lines in Fig. 1 b). However, one gets a clearer picture of the process by considering also the conditions at a given time (slanting lines in Fig. 1 b) or at a given V (horisontal lines in Fig. 1 b).

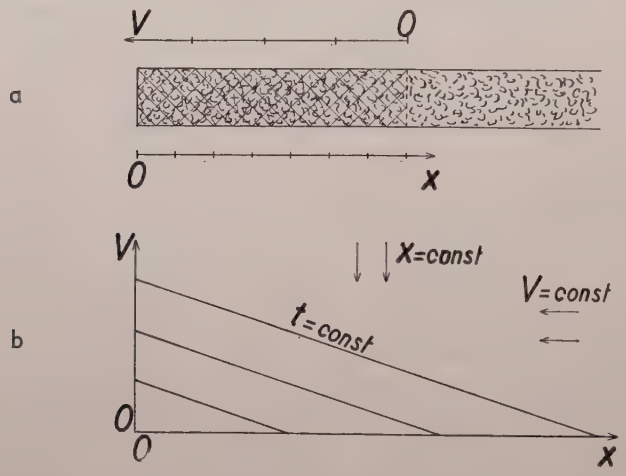

Fig. 1. a) Sorbent column with the two coordinates V (fixed relative to solution) and x (fixed relative to sorbent). b) V—x diagram showing the important types of section: $x =$ const., $V =$ const., and $t =$ const.

The following argument will also be valid for other types of packing, for instance with cylindrical or conical symmetry, or packing in a tube with varying diameter, provided only the problem can be treated as uni-dimensional. In such cases, as the variable x any quantity may be used that is proportional to the amount of sorbent that has been passed by the solution, such as the weight, or volume, or capacity of the sorbent passed. Of course, the amount sorbed (q, s_0) should in such case be expressed in corresponding units.

Although the word "solution" will consistently be used in the following, the results are also valid for the filtration of a gas through a sorbent.

Sorption: general case

We shall denote the various substances present in the solution by A_1, A_2, or in general A_i. At a certain point (x, V), the concentration of A_i in the solution is c_i mmol/ml, and the sorbed amount of A_i is q_i mmol/cm of sorbent; c_i and q_i can be considered as functions of x and V. When an element dx of the sorbent is passed by an element dV of the solution, the *conservation of matter* requires that the increase in $c_i\,dV$, $dx\left(\frac{\partial c_i}{\partial x}\right)_V dV$, be equal to the decrease in $q_i\,dx$, $-dV\left(\frac{\partial q_i}{\partial V}\right)_x dx$, which gives the well-known formula:

$$\left(\frac{\partial c_i}{\partial x}\right)_V + \left(\frac{\partial q_i}{\partial V}\right)_x = 0. \tag{1}$$

This formula must be valid for each substance A_i, independent of whether equilibrium is reached or not.

However a necessary condition is that there is no other process for transporting matter along the tube than the movement of the solution; thus diffusion is neglected.

If some of the solution is kept immobile in the pores of the sorbent, the solutes in it will be included in q. Thus our q corresponds to $Q + \alpha c = M f(c) + \alpha c$ in De Vault's (1943) equations — The amounts of A_i can, of course, be expressed in other units than mmol, for instance in g.

The *equilibrium* condition can be expressed by means of the sorption isotherm $q_i(c_i)$, which is strictly valid only when the substance A_i is the only sorbate present in the solution. It is normally convex to the q axis (Fig. 2 a). If several substances are present, q_i will in general be a complicated function of all the other c:s, too.

The Ψ condition

Our present task is to study the types of front obtained if equilibrium exists at every moment and everywhere between solution and sorbent. We shall find that very simple formulae are obtained in all such cases where *the c_i and q_i can be expressed as functions of the single variable*

$$\Psi = \frac{V}{x}. \tag{2}$$

This "*Ψ condition*" will be valid provided only the boundary conditions contain Ψ as the only variable, and not V or x separately:

$$\text{at } \Psi = 0 \quad (V = 0): \quad c_1' \, c_2' \ldots c_i' \ldots \quad q_1' \, q_2' \ldots q_i' \ldots \text{ all constant} \tag{3 a}$$

$$\text{at } \Psi = \infty \; (x = 0): \quad c_1'' \, c_2'' \ldots c_i'' \ldots \quad q_1'' \, q_2'' \ldots q_i'' \ldots \text{ all constant} \tag{3 b}$$

Condition (3 a) means that initially the whole sorbent is uniformly loaded with a set of concentrations q_i' of the sorbates; a sorbent with this load would be in equilibrium with a solution with the set of concentrations c_i'. One may imagine that the first layer of solution ($V = 0$) either runs into an originally dry sorbent with the said loads q_i', or that it displaces an originally present solution with the equilibrium concentrations c_i'. Condition (3 b) states that the solution entering the column at $x = 0$ always has the same composition c_i'', which corresponds to a set of values q_i''. In the following we shall consider a number of cases where these conditions are valid or can be made valid by a change in the origin of the coordinates.

If the Ψ condition is valid, then in formula (1):

$$\left(\frac{\partial q_i}{\partial V}\right)_x = x^{-1} \frac{d q_i}{d \Psi}; \quad \left(\frac{\partial c_i}{\partial x}\right)_V = -\Psi x^{-1} \frac{d c_i}{d \Psi}$$

which gives

$$\frac{d c_i}{d \Psi}\left(\frac{d q_i}{d c_i} - \Psi\right) = 0. \tag{4}$$

Equation (4) requires that

$$\text{either } c_i = \text{const.} \tag{4 a}$$

$$\text{or } \frac{d q_i}{d c_i} = \Psi \left(= \frac{V}{x}\right). \tag{4 b}$$

In many cases *sharp fronts* are obtained, which means that there are discontinuous jumps in the various q_i and c_i (see for example Fig. 3 or 4). Let us assume that the following values prevail on the two sides of a discontinuity at $\Psi = \Psi_s$

$$\Psi = \Psi_s - \varepsilon : \; q_i, \, c_i$$

$$\Psi = \Psi_s + \varepsilon : \; q_i + \triangle q_i, \; c_i + \triangle c_i$$

(ε = a very small quantity). If the Ψ condition is valid, Ψ_s should be a constant. When x_s increases by δx_s, $V_s = \Psi_s x_s$ increases by $\delta V_s = \Psi_s \delta x_s$. The amount of A_i that has been taken up by the sorbent, $\delta x_s \triangle q_i$, must be equal to the amount of A_i that has been given off by the solution, $\delta V_s \triangle c_i = \Psi_s \delta x_s \triangle c_i$, which gives the condition:

$$\Psi_s = \frac{\triangle q_i}{\triangle c_i} \text{ for a sharp front.} \tag{5}$$

The equations (4 a), (4 b), and (5) are the fundamental tools we need for the following.

Single solute: desorption

There is one single solute, A. Its sorption isotherm is given in Fig. 2 a; it is of the ordinary, convex type. The sorbent is originally loaded with q' (corresponding to c' at equilibrium), and pure solvent is introduced into the column ($c'' = 0$, $q'' = 0$). In this case, (4 b) can be realized only between the two Ψ values

$$\Psi_1 = \left(\frac{d\,q}{d\,c}\right)_{c = c'}; \quad \Psi_2 = \left(\frac{d\,q}{d\,c}\right)_{c = c'' = 0} \tag{6}$$

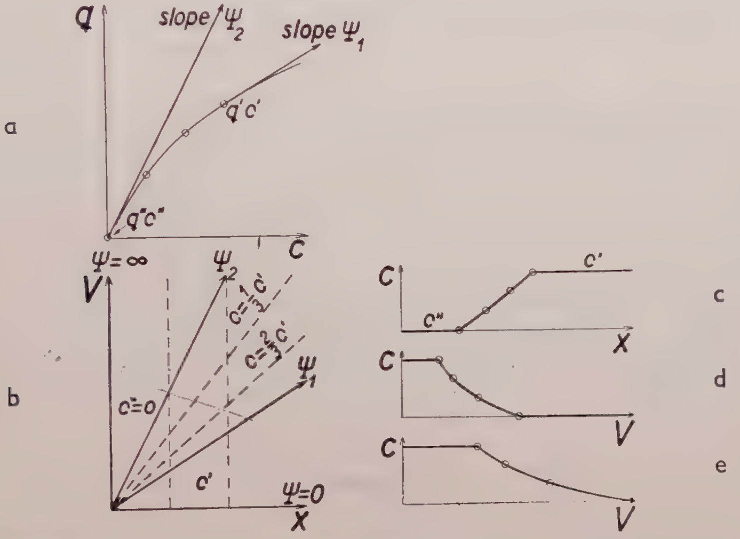

Fig. 2. Desorption of a single solute. Initial state: q', c'. Final state: $q'' = c'' = 0$. a) Sorption isotherm $q\,(c)$. b) V—x diagram. Thick lines with $\frac{V}{x} = 0$, Ψ_1, Ψ_2, and ∞ are drawn. Ψ_1 and Ψ_2 are the slopes of the tangents in Fig. 2 a. On the broken lines through the origin, $c = \frac{1}{3}\,c'$ and $\frac{2}{3}\,c'$. c) $c\,(x)$ for a given time (dotted slanting line in Fig. 2 b). d, e) $c\,(V)$ for two different x (broken vertical lines in Fig. 2 b). — The circles are at $c = 0$, $\frac{1}{3}\,c'$, $\frac{2}{3}\,c'$ and c'.

In Fig. 2 a, Ψ_1 and Ψ_2 are the slopes of the two tangents. Since in this case $\Psi_2 > \Psi_1$, the solution will have a lower concentration at higher Ψ and thus at higher V; this is as it should be for a desorption. The process will be described by:

$$\begin{array}{lll} \Psi \geqq \Psi_2 & c = c'' = 0, & q = q'' = 0 \\ \Psi_2 \geqq \Psi \geqq \Psi_1 & \dfrac{d\,q}{d\,c} = \Psi & \\ \Psi \leqq \Psi_1 & c = c', & q = q'. \end{array} \tag{7}$$

If the isotherm $q\,(c)$ is known, it is possible to calculate the complete course of $c\,(\Psi)$, and conversely, if the course of $c\,(\Psi)$ is known the isotherm can be calculated, as will be discussed in Part V.

Fig. 2 b is a diagram with x and V as coordinates ("$V-x$ diagram"), where the lines with $\Psi = 0$, Ψ_1, Ψ_2 and ∞ are plotted. The state of the column at a certain moment is represented by the dotted slanting line in Fig. 2 b and by Fig. 2 c, which gives the concentration c as a function of the distance x from the beginning of the column. Figs. 2 d and 2 e give, for two columns of different length x, the concentration c in the outflowing solution as a function of V; these curves correspond to vertical sections in Fig. 2 b (broken lines). In the following examples, the lines t = const, and x = const in the $V-x$ diagram will be omitted. For convenience, sections with V = const will often be given instead of those with t = const; they are very similar in appearance.

It should be noted that for any line through the origin, all points have the same values for Ψ and thus, because of the Ψ condition, the same values for q and c.

Obviously the "front" between Ψ_1 and Ψ_2 will become increasingly broader with increasing x. The general picture of this broadening desorption (elution) front will be the same even if c'' is not exactly zero, provided only $c'' < c'$.

Single solute: sorption

We assume that the sorbent is originally free from sorbate ($q' = c' = 0$), and that a solution of concentration c'' (corresponding to equilibrium with q'') is passed through the column. In this case, there can be no region with a continuously varying c, as can easily be seen from the following argument. If there were such a region, we find by differentiation of (4 b)

$$\frac{d\Psi}{dc} = \frac{d^2 q}{dc^2}.$$

Now, because of the bend of the isotherm, $\frac{d^2 q}{dc^2}$ is negative. On the other hand, in a sorption process c should increase with Ψ, whence $d\Psi/dc$ is positive. Thus a front with continuously varying c is not possible, and c must decrease by a sharp jump at a certain Ψ_s. The value for Ψ_s is found from (5):

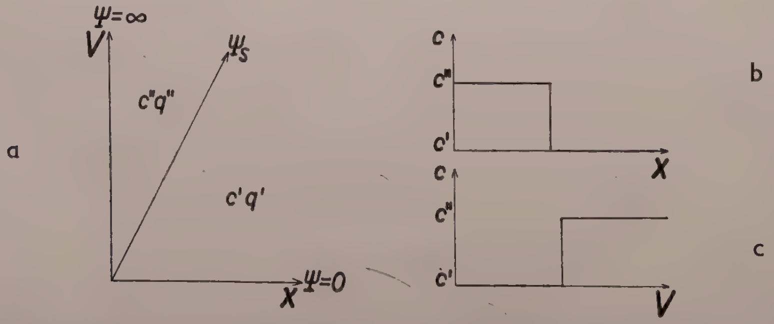

Fig. 3. Sorption of a single solute. Initial state: $q' = c' = 0$. Final state: q'' c''. a) $V-x$ diagram. b) $c\,(x)$ for constant t or V. c) $c\,(V)$ for a given x.

$$\Psi_s = \frac{q'' - q'}{c'' - c'} = \frac{q''}{c''}. \tag{8}$$

The process in the column is thus described by:

$$\begin{aligned} \Psi > \Psi_s, \quad & c = c'', \qquad q = q'' \\ \Psi < \Psi_s, \quad & c = c' = 0 \quad q = q' = 0. \end{aligned} \tag{9}$$

The $V - x$ diagram is given in Fig. 3 a, $c(x)$ at a constant time (or V) in Fig. 3 b, and $c(V)$ at a constant x in Fig 3 c.

A sharp front will also be obtained for other values of c' than 0, provided that $c' < c''$.

Sorption of several solutes (frontal analysis)

We assume that the column has originally no load (all $q_i' = c_i' = 0$), and that a solution of three substances, A_1, A_2 and A_3 is passed through it (concentrations c_1'', c_2'', and c_3''). The sorbability is assumed to increased from A_1 to A_3. The results for three substances can easily be generalized to any number of solutes. — In this case, as for a single solute, sharp fronts are obtained, one for each substance. Their Ψ values are given by equation (5):

$$\begin{aligned} \Psi_{s1} &= \frac{q_{11}}{c_{11}} \\ \Psi_{s2} &= \frac{q_{22}}{c_{22}} = \frac{q_{11} - q_{12}}{c_{11} - c_{12}} \\ \Psi_{s3} &= \frac{q_3''}{c_3''} = \frac{q_{22} - q_2''}{c_{22} - c_2''} = \frac{q_{12} - q_1''}{c_{12} - c_1''}. \end{aligned} \tag{10}$$

The symbols for the concentrations, c_{11} etc., are explained by the $V - x$ diagram in Fig. 4 a. In Fig. 4 b, $c(x)$ is shown for a given V, and in Fig. 4 c $c(V)$ for a given x.

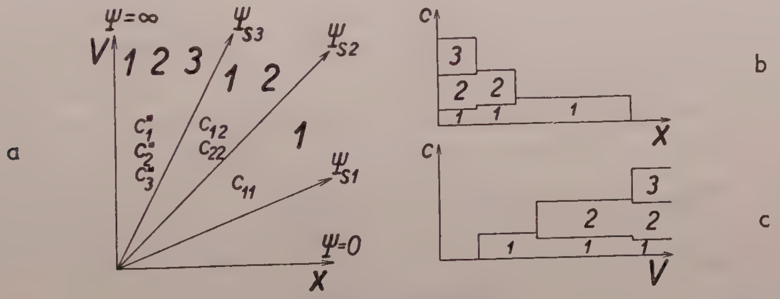

Fig. 4. Sorption of (frontal analysis of) a mixture of three solutes. a) V—x diagram. b) $c(x)$ for a given V. c) $c(V)$ for a given x. The concentrations are accumulated; the concentration of each substance is given by the height of the corresponding area.

In a single sorption experiment with one column, one can determine the three Ψ_{si} and the six c:s. With the aid of the six equations (10) it is thus possible in principle to determine the six q:s.

This procedure — "frontal analysis" — was first devised by TISELIUS (1940 a, b) as a method for analysing mixtures. Equations analogous to (10) have been derived by DE VAULT (1943) and by CLAESSON (1946), from arguments on the conservation of matter in the column.[1] It should be noted, however, that they are obtained here automatically from equation (5) and the Ψ condition, thus saving a certain amount of brain work. — The quantities Ψ_{si} are identical with the "specific retention volumes" of TISELIUS (1941), if V is expressed in ml and x in g of sorbent.

The same way of reasoning, using the Ψ condition, can be applied also to other cases, where several solutes take part in a sorption process. However, we shall prefer to leave the general case for a moment and consider instead the case of ion exchange, where the interaction of the different sorbates is somewhat easier to grasp.

Exchange sorption (ion exchange)

Let us assume that our sorbent is a cation exchanger with a constant sorption capacity for cations, s_0 mF (millifaradays or mval) per cm. During the process of ion exchange, the total concentration of cations in the solution will then be constant; let us call it a_0 mF/ml. The cations in the solution are all, we assume, univalent and are denoted by A_1^+, A_2^+, ... in general A_i^+. Sometimes for brevity we shall call them ions 1, 2, ... i etc. We shall find it convenient to introduce the quantities α_i and β_i, defined by

$$c_i = \alpha_i a_0; \quad q_i = \beta_i s_0. \tag{11}$$

It follows from our assumptions:

$$\Sigma \alpha_i = 1; \quad \Sigma \beta_i = 1. \tag{12}$$

If multivalent cations are also present, their concentrations should be expressed in mval; moreover the expression (14) below for the equilibrium quotient will be different, and will in general depend much more on a_0 than the expression for univalent ions. However, the following conclusions are valid also for multivalent cations, provided their adsorption isotherms, $\beta(\alpha)$, follow the few general rules set down below.

The law of the conservation of matter (1) after insertion of (11) gives

$$a_0 \frac{\partial \alpha_i}{\partial x} + s_0 \frac{\partial \beta_i}{\partial V} = 0 \tag{13 a}$$

or

$$\frac{\partial \alpha_i}{\partial (s_0 x)} + \frac{\partial \beta_i}{\partial (a_0 V)} = 0. \tag{13 b}$$

---

[1] These authors used difference formulae with $\frac{\Delta V}{\Delta x}$ where we have $\Psi = \frac{V}{x}$.

We shall denote the equilibrium quotient for the reaction ($R^- =$ fixed anion of exchanger)

$$A_i^+ + A_k R \rightleftarrows A_k^+ + A_i R$$

$$\alpha_i a_0 \quad \beta_k s_0 \quad \alpha_k a_0 \quad \beta_i s_0$$

by

$$\varkappa_{ik} = \frac{\beta_i \alpha_k}{\beta_k \alpha_i} = \varkappa_{ki}^{-1}. \tag{14}$$

Generally, $\varkappa_{ik}$ is not a constant. If it were, and if the two ionic species A_i^+ and A_k^+ were alone in the solution, the sorption isotherm of A_i^+, $\beta_i(\alpha_i)$ would take the form:

$$\frac{\beta_i (1 - \alpha_i)}{(1 - \beta_i)\, \alpha_i} = K_{ik} = \text{const.} \tag{14 a}$$

Even if this is not exactly true, the isotherm $\beta(\alpha)$ in most cases retains the general shape of equation (14 a) (Fig. 5). Of course, at $\alpha_i = 0$, $\beta_i = 0$, and at $\alpha_i = 1$, $\beta_i = 1$. Moreover, the curve generally keeps on one side of the line $\beta = \alpha$; either $\beta_i > \alpha_i$ for all α_i except 0 and 1, or $\beta_i < \alpha_i$ for all α_i except 0 and 1. (For an exception, see GLUECKAUF 1947.)

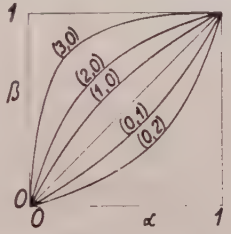

Fig. 5. Isotherms $\beta(\alpha)$ for a number of combinations of the ions 0, 1, 2, and 3 on an ion exchanger.

We shall assume that we can arrange all our cations in a series of increasing sorbability: $A_0^+ \; A_1^+ \; A_2^+ \ldots A_i^+ \ldots$, where a cation with a *higher index* i is always *more strongly sorbed* than one with a lower index. This can be expressed in the following way. Let (i, k) be the curve $\beta_i(\alpha_i)$ in the case where only A_i^+ and A_k^+ are present. Then, using A_0^+ as a reference ion, the curves $(1, 0)$, $(2, 0) \ldots (i, 0) \ldots$ should form a single family of curves, which do not intersect at any point except at 0 and 1 (Fig. 5). It will, moreover, be assumed that the mutual influence of the ions is such that the point $\beta_0(\alpha_0)$ in any mixture of A_0^+, A_1^+, and A_2^+ will, for instance, be in between the curves $(0, 1)$ and $(0, 2)$.

It will be noted that (k, i) is exactly the inversion of (i, k) with the centre of symmetry in $\frac{1}{2}, \frac{1}{2}$.

The equilibria of ion exchangers will be discussed more fully in subsequent papers by EKEDAHL and HÖGFELDT.

The Ψ condition

In the mathematical treatment of ion exchange columns, it is advantageous to replace Ψ by the quantity ψ, which is proportional to Ψ:

$$\psi = \frac{a_0 V}{s_0 x} = \frac{a_0}{s_0} \Psi. \tag{15}$$

Here ψ has a very simple meaning: it is the ratio between $a_0 V$, the total amount of cations that have passed the layer at x, and $s_0 x$, the total capacity of the ion exchanger between the inlet end of the column ($x = 0$) and the layer at x.

The condition that all the α_i and β_i are functions of Ψ only — or, which is the same, of ψ only — is just like before, that the sorbent has originally a uniform load and that, from $V = 0$ onwards, the solution entering the column always has the same composition. (In this case one can imagine that the solution of $V = 0$ either flows into a dry sorbent or that it replaces a layer of pure solvent.) The conditions can be expressed:

$$\text{at } \psi = 0 \quad (V = 0) \quad \alpha_1' \; \alpha_2' \; \ldots \; \alpha_i' \; \ldots \; \beta_1' \; \beta_2' \; \ldots \; \beta_i' \; \ldots \text{ all constant} \tag{16 a}$$

$$\text{at } \psi = \infty \, (x = 0) \quad \alpha_1'' \; \alpha_2'' \; \ldots \; \alpha_i'' \; \ldots \; \beta_1'' \; \beta_2'' \; \ldots \; \beta_i'' \; \ldots \text{ all constant.} \tag{16 b}$$

Equation (4) gives, after insertion of (11) and (15)

$$\frac{d\,\alpha_i}{d\,\psi}\left(\frac{d\,\beta_i}{d\,\alpha_i} - \psi\right) = 0 \tag{17}$$

which means that

$$\text{either } \alpha_i = \text{const.} \tag{17 a}$$

or

$$\frac{d\,\beta_i}{d\,\alpha_i} = \psi. \tag{17 b}$$

For a sharp front we find, using (5), (11), and (15)

$$\psi_s = \frac{\triangle\,\beta_i}{\triangle\,\alpha_i}. \tag{18}$$

One sort of ions added

If the column is originally saturated with A_2^+, and A_1^+ is introduced at the end of the column, the conditions will be analogous to those for the "desorption" case already considered. Equation (17 b) will be valid between the limits:

$$\psi_1 = \left(\frac{d\,\beta_1}{d\,\alpha_1}\right)_{\alpha_1=0}; \quad \psi_2 = \left(\frac{d\,\beta_1}{d\,\alpha_1}\right)_{\alpha_1=1} = \left(\frac{d\,\beta_2}{d\,\alpha_2}\right)_{\alpha_2=0}. \tag{19}$$

As illustrated in Fig. 6 a, ψ_1 and ψ_2 are the slopes of the tangents at the zero ends of the equilibrium curves (1, 2) and (2, 1). If an ideal isotherm (14 a) is valid, $\psi_1 = K_{12}$ (< 1), and $\psi_2 = K_{21} = K_{12}^{-1}$ (> 1).

Fig. 6 b shows the $a_0 V - s_0 x$ diagram for this case, Fig. 6 c shows α_1 as a function of $s_0 x$ for constant time, and figs. 6 d and e show α_1 as a function of $a_0 V$ for two given values of x.

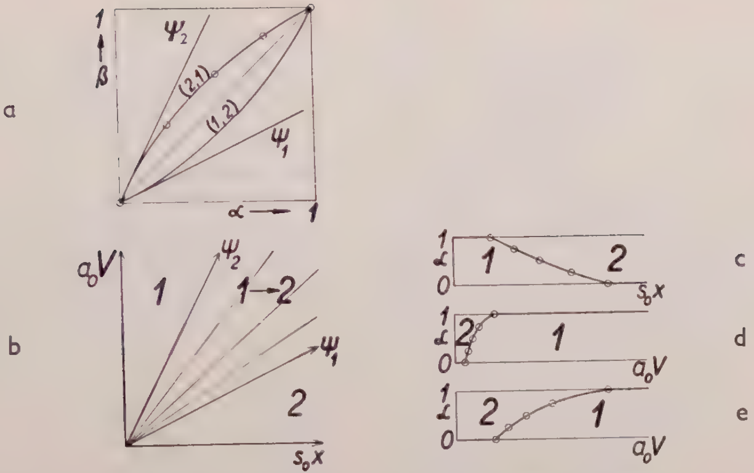

Fig. 6. a) Isotherms $(1, 2) = \beta_1(\alpha_1)$ and $(2, 1) = \beta_2(\alpha_2)$ for the two ions 1 and 2 on an ion exchanger. The curves are symmetrical about (0.5, 0.5). The slopes of the tangents at the zero ends are ψ_1 and ψ_2. b) Replacing ion 2 by 1 (which is less strongly sorbed): $a_0 V - s_0 x$ diagram. c) $\alpha(s_0 x)$ for a given V. d, e) $\alpha(a_0 V)$ for two given values of x. In c—e, the height of each area at a certain point gives the corresponding α. For comparison with Fig. 2 c—e, the curves in Fig. 6 c—e, should be turned upside down and α_2 compared to c.

If, on the contrary, the column is saturated with A_1^+ ($\beta_1' = 1$), and A_2^+ is introduced ($\alpha_2'' = 1$), the conditions will be analogous to those for the sorption case above. There can be no region with a continuous $\alpha(\psi)$, since (17 b) would give $\frac{d\psi}{d\alpha_1} = \frac{d^2\beta_1}{d\alpha_1^2}$, where the left term is negative, and the right one positive. Thus we get a sharp front, the position of which is given by (18)

$$\psi_s = 1. \tag{20}$$

This case is shown in Fig. 7 a—c.

We can draw the general conclusion, that if one sort of ion is replaced by another, we get a *sharp front, if the new ion is sorbed more strongly than the old* one, and that we get a *broadening front, if the new ion is less strongly sorbed* than the old one.

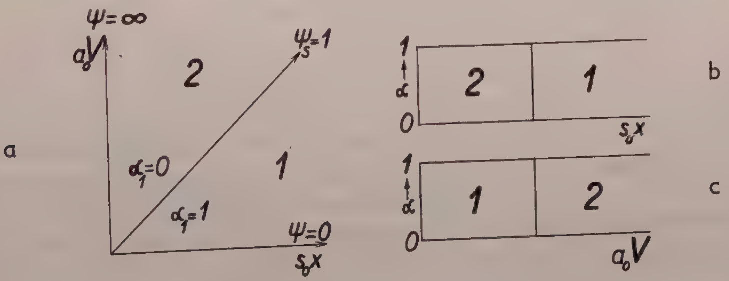

Fig. 7. Replacing ion 1 by ion 2 (which is more strongly sorbed). a) $a_0 V - s_0 x$ diagram. b) $\alpha(s_0 x)$ for a given V. c) $\alpha(a_0 V)$ for a given x.

It is easily seen that it is not a necessary condition that the boundary values of α_1 be either 0 or 1. If A_2^+ is sorbed more strongly than A_1^+, we shall always get a sharp front if $\alpha_2'' > \alpha_2'$, and a broadening front if $\alpha_1'' > \alpha_1'$.

Several sorts of ions added

Let us assume that the sorbent is initially saturated with A_0^+, and that the incoming solution contains a mixture of the three ions A_1^+, A_2^+, and A_3^+, which are thus according to our convention all sorbed more strongly than is A_0^+. In this case we get three sharp fronts, as is shown in Fig. 8. For $\psi > \psi_{s3}$ we have the incoming solution with concentrations α_1'', α_2'', and α_3''. Between ψ_{s3} and ψ_{s2} we have a solution with only A_1^+ and A_2^+ (α_{11}, α_{21}), between ψ_{s2} and ψ_{s1} we have pure A_1^+, and at $\psi < \psi_{s1}$ there is pure A_0^+. For the positions of the fronts we find immediately from equation (18)[1]

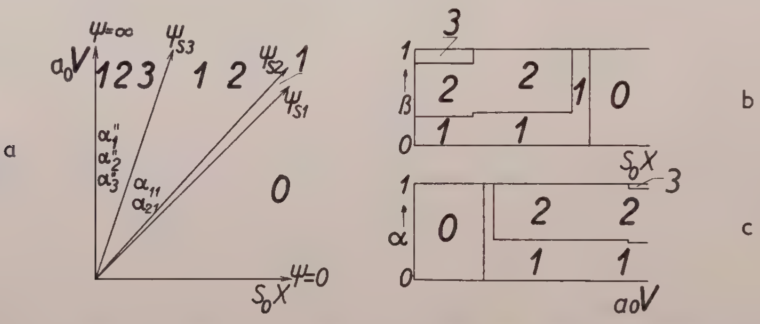

Fig. 8. Addition of three sorts of ions (frontal analysis). a) a_0V—s_0x diagram. b) $\beta\,(s_0x)$ for a given V. c) $\alpha\,(a_0V)$ for a given x. The same concentrations have been assumed as in Fig. 13.

$$\psi_{s1} = 1$$

$$\psi_{s2} = \frac{\beta_{21}}{\alpha_{21}} = \frac{1-\beta_{11}}{1-\alpha_{11}}$$

$$\psi_{s3} = \frac{\beta_3''}{\alpha_3''} = \frac{\beta_{21}-\beta_2''}{\alpha_{21}-\alpha_2''} = \frac{\beta_{11}-\beta_1''}{\alpha_{11}-\alpha_1''}. \tag{21}$$

It will make no difference in our calculations if A_0^+ is identical with A_1^+. This occurs for instance if an acid solution of a number of cations is introduced into an ion exchanger that is originally saturated with H^+. In this case, too, sharp fronts are obtained, provided the ions added are sorbed more strongly than $A_0^+ = H^+$.

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[1] Because of (12), (21) contains only four independent equations.

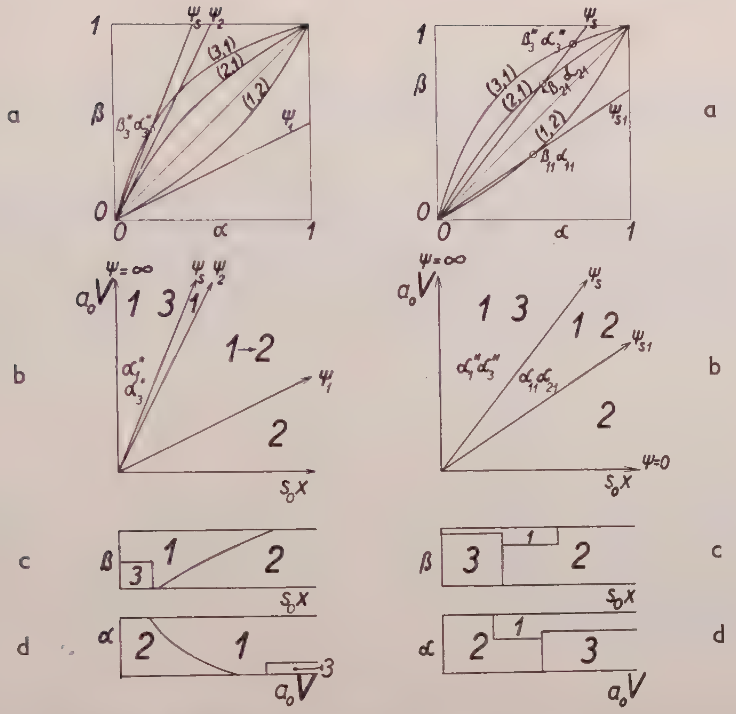

Fig. 9 and 10. Replacing ion 2 by a mixture of 3 and 1 in two different cases (small and large α_3''). a) Isotherms (3, 1), (2, 1), and (1, 2). b) $a_0 V - s_0 x$ diagrams. c) $\beta (s_0 x)$ for a given V. d) $\alpha (a_0 V)$ for a given x.

We shall now assume that the ion originally sorbed is A_2^+, and that a mixture of A_1^+ and A_3^+ is introduced, thus one ion (A_1^+) which is less strongly sorbed than A_2^+, and one which is more strongly sorbed. Then two cases can occur, as is shown in Figs. 9 and 10. For small concentrations of A_3^+, a front with continuously varying α_2 is obtained between ψ_1 and ψ_2, and A_3^+ forms a sharp front at ψ_s (Fig. 9). The ψ values are given by equations (17 b) and (18):

$$\psi_1 = \left(\frac{d\,\beta_1}{d\,\alpha_1}\right)_{\alpha_1=0}; \quad \psi_2 = \left(\frac{d\,\beta_1}{d\,\alpha_1}\right)_{\alpha_1=1}; \quad \psi_s = \frac{\beta_3''}{\alpha_3''}; \quad (\psi_s > \psi_2). \tag{22}$$

Of these quantities, ψ_1 is always < 1, whereas both ψ_2 and ψ_s are > 1. The three quantities are given in Fig. 9 a: ψ_1 and ψ_2 are the slopes of the zero tangents of the isotherms (1, 2) and (2, 1). If the point (β_3'', α_3'') is situated to the left of the ψ_2 line, ($\psi_s > \psi_2$), the case in Fig. 9 is realized. Otherwise this is impossible, and we get the picture in Fig. 10.

In this second case we have two sharp fronts. Between the incoming solution and the pure A_2^+ we have a region with A_1^+ and A_2^+ at constant concentrations (α_{11} and α_{21}). The positions ψ_s and ψ_{s1} of the two fronts are given by equation (18).

$$\psi_s = \frac{\beta_3''}{\alpha_3''} = \frac{\beta_{21}}{\alpha_{21}}; \quad \psi_{s1} = \frac{1-\beta_{21}}{1-\alpha_{21}} = \frac{\beta_{11}}{\alpha_{11}}. \tag{23}$$

Fig. 10 a shows how ψ_{s1} can be obtained by a geometrical construction by means of the isotherms (3, 1), (2, 1), and (1, 2).

From the following argument it may be seen that if $\psi_s < \psi_2$ in (22) no other type of front than that in Fig. 10 is possible. First of all, α_3 can only vary in discontinuous steps, since β_3 (α_3) is convex upwards in all mixtures of 3, 1, and 2. One might imagine, that the region where α_2 varies continuously would extend up to the point where it meets a sharp front of A_3^+. Let us assume that this is so, and that the values of β_2 and α_2 are β_{2s} and α_{2s} on the right side of the front (lower ψ), and β_{21} and α_{21} on the left side (higher ψ). Then, according to (17 b) and (18)

$$\psi_{1s} = \left(\frac{d\beta_2}{d\alpha_2}\right)_{\alpha_{2s}} = \frac{\beta_{2s} - \beta_{21}}{\alpha_{2s} - \alpha_{21}}.$$

This means that the point $(\beta_{21}, \alpha_{21})$ is situated on the tangent to the isotherm (2, 1) at the point $(\beta_{2s}, \alpha_{2s})$. But now the point $(\beta_{21}, \alpha_{21})$ must, according to our assumptions, be situated to the right of (2, 1) since behind the front a certain amount of A_3^+ is present. Thus the tangent cannot go through $(\beta_{21}, \alpha_{21})$, and there can be no solution where a line with varying α_2 will meet the region with A_3^+. The only remaining possibility is an intermediate region with constant concentrations, say $\alpha_{11}, \alpha_{21}, \alpha_{31}$. For the ψ value of the front between this region and the region of pure A_2^+ we find

$$\psi_{s1} = \frac{\beta_{31}}{\alpha_{31}} = \frac{\beta_{11}}{\alpha_{11}}$$

which is possible only if either α_{11} or α_{31} is zero, since otherwise the points β_{31}, α_{31} and β_{11}, α_{11} will be on different sides of the line $\beta = \alpha$. Thus we are led to the picture in Fig. 10 as the only acceptable one.

Displacement of a mixture of ions

Mixture of two sorts of ions

Let the sorbent originally consist of two homogeneous parts. Between $x = 0$ and $x = x_0$ it is loaded with a mixture of A_1^+ and A_2^+ $(\beta_1^0 + \beta_2^0 = 1)$. From x_0 on to infinity the ion exchanger is saturated with A_0^+. Now from $x = 0$ we introduce a solution of the ion A_N^+, which is more strongly sorbed than any of the other ions. This case would be analogous to that of separation of ions by displacement, although in most practical cases a certain separation of the ions has already occurred before the displacing ion is added.

The Ψ condition can be applied to the process starting at the end $(x = 0, V = 0)$. Two sharp fronts are formed, one between A_N^+ and A_2^+, and one between A_2^+ and a mixture of A_1^+ and A_2^+. For convenience these fronts will be referred to in the following as front N–2 and front 2–12; other fronts will be denoted analogously. For the ψ values of the fronts we find with (18) front N–2 $$\psi = \frac{a_0 V}{s_0 x} = 1$$

front 2–12: $$\psi = \frac{a_0 V}{s_0 x} = \frac{\beta_1^0}{\alpha_1^0} \quad (< 1). \tag{24 a}$$

But we can also apply the Ψ condition to the process at $x = x_0$ and larger x, if we move our origin of coordinates to $(x = x_0, V = 0)$. Originally, the sorbent at $x \geqq x_0$ is uniformly loaded, and the solution that enters at $x = x_0$ is also of uniform composition (α_1^0, α_2^0). Using the symbol ψ' for ψ values counted from the new origin we find that there are two more fronts originating from x_0:

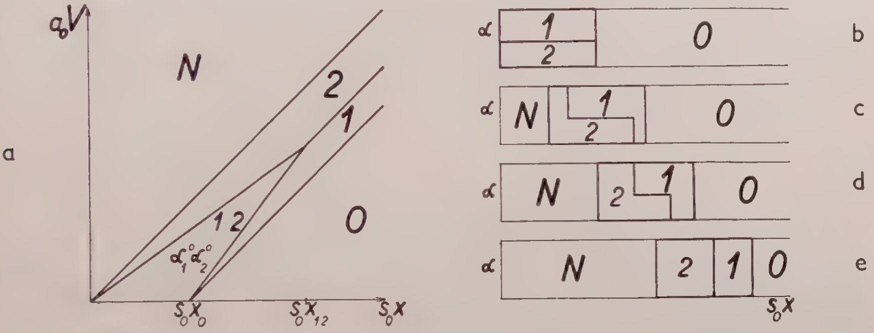

Fig. 11. Displacement separation of a mixture of ions 1 and 2 between ions O and N. a) a_0V—s_0x diagram, b—e) $\alpha\,(s_0x)$ at four different V.

front 12–1: $$\psi' = \frac{a_0 V}{s_0\,(x - x_0)} = \frac{\beta_2^0}{\alpha_2^0} \quad (> 1)$$

front 1–0: $$\psi' = \frac{a_0 V}{s_0\,(x - x_0)} = 1. \tag{24 b}$$

The $a_0 V - s_0 x$ diagram in Fig. 11 a, and the $\alpha\,(s_0 x)$ curves in Fig. 11 b–e show that the two fronts 2–12 and 12–1 will meet at a certain point (x_{12}, V_{12}); at this point the mixed region disappears. From the equations of the lines we find

$$\frac{s_0 x_{12}}{s_0 x_0} = \frac{\beta_2^0\,\alpha_1^0}{\alpha_1^0 - \beta_1^0} = \frac{1}{1 - \varkappa_{12}} = \frac{\varkappa_{21}}{\varkappa_{21} - 1}$$

$$\frac{a_0 V_{12}}{s_0 x_0} = \frac{\beta_1^0\,\beta_2^0}{\alpha_1^0 - \beta_1^0} = \frac{\varkappa_{21}}{\varkappa_{21} - 1} - \beta_2^0. \tag{24 c}$$

where $\varkappa_{12} = \varkappa_{21}^{-1}$ is the equilibrium quotient in the mixture according to equation (14).

From the point (x_{12}, V_{12}) on, we get a new front 2–1, for which the Ψ condition applies, if we choose this point as the origin of coordinates. We thus get:

front 2–1:
$$\frac{a_0\,(V - V_{12})}{s_0\,(x - x_{12})} = 1. \tag{24 d}$$

If we want a complete separation of A_1^+ and A_2^+, x_{12} is obviously the *minimum length* of the column. On the other hand we cannot gain anything by making the column much longer than x_{12}.

The *l–y* diagram

In a process of displacement of ions like that described above, the only region that is of interest is the one of constant length between the regions of A_N^+ and A_0^+. We can get a more compact picture of the process than is given by the $a_0\,V - s_0\,x$ diagram by choosing the new coordinates.

$$l = \frac{s_0\,x}{s_0\,x_0}; \qquad y = \frac{s_0\,x - a_0\,V}{s_0\,x_0} \tag{25 a}$$

thus

$$s_0\,x = s_0\,x_0\,l; \qquad a_0\,V = s_0\,x_0\,(l - y). \tag{25 b}$$

For a front in the $a_0\,V - s_0\,x$ diagram, starting at the point $(s_0\,x',\ a_0\,V')$, we find from the Ψ condition and (18)

$$\frac{\triangle\,\beta_i}{\triangle\,\alpha_i} = \frac{a_0\,(V - V')}{s_0\,(x - x')}. \tag{26}$$

If we transform the equation of the front to the new coordinates l and y, we find (the starting point is l', y').

$$\frac{l - l'}{y - y'} = \frac{\triangle\,\alpha_i}{\triangle\,\alpha_i - \triangle\,\beta_i}. \tag{27}$$

By applying equation (27) to the case considered above, we find

Front N–2: $y = 0$

Front 2–12: $l\,(\alpha_1^0 - \beta_1^0) = y\,\alpha_1^0$

Front 12–1: $(l - 1)\;(\alpha_2^0 - \beta_2^0) = (y - 1)\,\alpha_2^0$

Front 1–0: $y = 1$

Front 2–1: $y = y_{12}$. (28 a)

The fronts 2–12 and 12–1 will coincide at the point

$$y_{12} = \beta_2^0; \quad l_{12} = \frac{\alpha_1^0\,\beta_2^0}{\alpha_1^0 - \beta_1^0} = \frac{1}{1 - \varkappa_{12}} = \frac{\varkappa_{21}}{\varkappa_{21} - 1}. \tag{28 b}$$

$\varkappa_{21} = \varkappa_{12}^{1}$ is as before the equilibrium quotient at the concentrations prevailing in the original mixture. In Fig. 12 a, the $l - y$ diagram has been based on the following figures: $\beta_1^0 = 0.4$ $\beta_2^0 = 0.6$, $\alpha_1^0 = 0.55$, $\alpha_2^0 = 0.45$; from which we calculate $\varkappa_{12} = \frac{6}{11}$, $l_{12} = 2.2$. The same values were used for Fig. 11 a. Fig. 12 b–e shows four subsequent stages of the separation of the mixture (l values 0.7, 1.0, 1.6, and > 2.2). In this case, the minimum length of the column for a complete separation is $2.2\ x_0$.

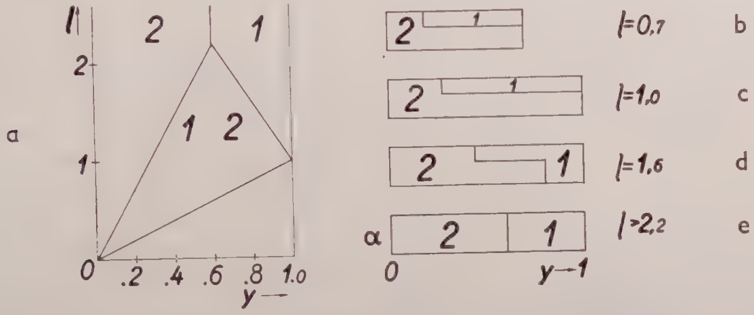

Fig. 12. a) $l - y$ diagram for the same displacement process as in Fig. 11, b—e) α (y) for four different l. — The area below the line $l = y$ is not real; it would correspond to negative V.

Obviously, the minimum length is not influenced by the nature of the two ions A_N^- and A_0^+, provided that the former is more strongly sorbed and the latter is less strongly sorbed than any ion in the mixture to be separated.

Mixture of three sorts of ions

In Fig. 13 a is given the $l - y$ diagram for the separation of a mixture of three sorts of ions, A_1^-, A_2^-, and A_3^-, through displacement between A_N^+ and A_0^-. Starting from the point (0, 0) we get two fronts 3–23 and 23–123; from (1, 1) two other fronts 123–12 and 12–1 develop. At the point (l_2, y_2) the region 123 disappears. From this point onwards, a region with pure 2 wedges in between 23 and 12. These two binary mixtures disappear at (l_{23}, y_{23}) and (l_{12}, y_{12}). We shall denote the α values by α_1^0, α_2^0, and α_3^0 in 123, by α_1 and $1 - \alpha_1$ in 12, and by α_3 and $1 - \alpha_3$ in 23; the β values are denoted in an analogous way. By applying equation (27) we find the following equations; for the sake of uniformity we have not changed the signs in those equations where the coefficients are negative.

Front 3–23:
$$l(\beta_3 - \alpha_3) = y(1 - \alpha_3)$$

Front 23–123:
$$\frac{l}{y} = \frac{\alpha_1^0}{\alpha_1^0 - \beta_1^0} = \frac{\alpha_3 - \alpha_3^0}{\alpha_3 - \alpha_3^0 - \beta_3 + \beta_3^0}$$

Front 123–12:
$$\frac{l - 1}{y - 1} = \frac{\alpha_3^0}{\alpha_3^0 - \beta_3^0} = \frac{\alpha_1 - \alpha_1^0}{\alpha_1 - \alpha_1^0 - \beta_1 + \beta_1^0} \tag{29}$$

Front 12–1: $(l-1)(\beta_1-\alpha_1)=(y-1)(1-\alpha_1)$

Front 23–2: $(l-l_2)(\alpha_3-\beta_3)=(y-y_2)\alpha_3$

Front 2–12: $(l-l_2)(\alpha_1-\beta_1)=(y-y_2)\alpha_1$

Front 3–2: $y=y_{23}$

Front 2–1: $y=y_{12}\,.$ (29)

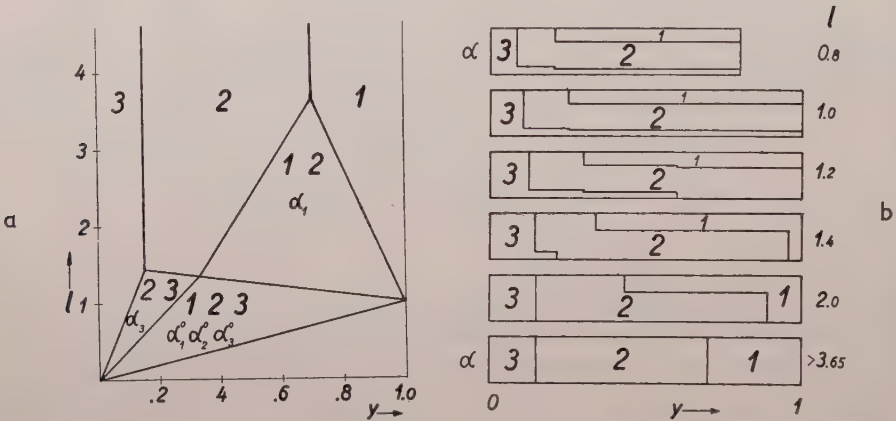

Fig. 13. Separation of a mixture of three ions 1, 2 and 3 between 0 and N. a) l—y diagram. The points where the areas 23, 123, and 12 disappear are (l_{23}, y_{23}), (l_2, y_2), and $(l_{12}\ y_{12})$. b) $\alpha\,(y)$ diagrams at six different values for l.

The points of intersection: (l_2, y_2) etc. are obtained from the formulae for the corresponding two fronts; in order to save space we shall not give the solutions explicitly. As might have been expected, we find

$$y_{23}=\beta_3^0;\quad y_{12}=1-\beta_1^0$$

after the separation is complete, each ion thus occupies a volume of the solution (or length of the column) which is proportional to its molar fraction in the initial mixture.

Fig. 13 a has been constructed from a hypothetical case assuming $\beta_1^0=0.30$, $\beta_2^0=0.55$, and $\beta_3^0=0.15$, $K_{32}=3$, $K_{12}=0.75$; the equilibrium constants have been assumed to be really constant. From the equilibrium conditions we find $\alpha_1^0=0.40$, $\alpha_2^0=0.55$, and $\alpha_3^0=0.05$. The equations of the front 23–123, together with K_{32}, gives $\beta_3=0.156$, $\alpha_3=0.058$, and the equations of 123–12 and K_{12} give $\beta_1=0.351$, $\alpha_1=0.417$.

The region 123 disappears at $y_2=0.333$, $l_2=1.333$ (intersection of 23–123 and 123–12) 23 disappears at $y_{23}=0.15$, $l_{23}=1.44$ (intersection of 3–23 and $y=0.15$); and 12 disappears at $y_{12}=0.70$, $l_{12}=3.65$ (intersection of 12–1 and $y=0.70$). The minimum length of the column necessary to separate A_3^+ completely from the other ions is then 1.44 x_0; to separate A_1^+ and A_2^+ completely also, the column should be at least 3.65 x_0.

Fig. 13 b shows subsequent stages in the development in the form of $\alpha(y)$ diagrams for $l = 0.8$, 1, 1.2, 1.4, 2, and > 3.65.

The argument given here can easily be generalized to the separation of a larger number of ions.

The same type of argument can also be applied to *separation by displacement* in the *general case* of non-exchange sorption. The A_0^+ region in front of the separating mixture corresponds in this case to a region of pure sorbent without any load. In the final state, all the different substances are separated and arranged in the order of their sorbability. If we consider the front between two subsequent substances A_i and A_k, and choose as the origin of coordinates the position of the front at any moment, the Ψ condition and (5) give immediately

$$\frac{q_i}{c_i} = \frac{q_k}{c_k}. \tag{30}$$

By passing from this front to the next one etc. we easily see that the ratio q/c must be the same for all substances. This condition has been given by TISELIUS (1943) who was the first to see the advantages of displacement analysis. A formally analogous case is met with in the case of separation of ions on an ion exchanger, when the solution of A_N^+, and the mixture to be separated also contain A_0^+ (which is often H^+). In this case for all ions except A_0^+

$$\frac{\beta_i}{\alpha_i} = \frac{\beta_N}{\alpha_N}. \tag{31}$$

A free band

The case of a "free band" of solute can also be treated by means of the Ψ condition. Let us assume that initially the sorbent between $x = 0$ and x_0 contains a sorbate at a concentration q' (corresponding to c' at equilibrium), that the rest of the column is pure sorbent, and that pure solvent is passed through the column from $V = 0$.

Using $(x = 0,\ V = 0)$ as the origin, we can apply the Ψ condition and find, just as with equation (6), that we get a front with continuously varying c between the two Ψ values

$$\Psi_1 = \frac{V}{x} = \left(\frac{dq}{dc}\right)_{c'}; \qquad \Psi_2 = \frac{V}{x} = \left(\frac{dq}{dc}\right)_0. \tag{32}$$

On the other hand, the Ψ condition can also be applied using $(x = x_0,\ V = 0)$ as the origin. Starting from this origin we get a sharp front, for which (5) gives

$$\Psi_s = \frac{V}{x - x_0} = \frac{q'}{c'}. \tag{33}$$

As is seen from the $V-x$ diagram, Fig. 14 a, the two fronts Ψ_1 and Ψ_s will intersect at a certain point. From this point onwards, the total amount

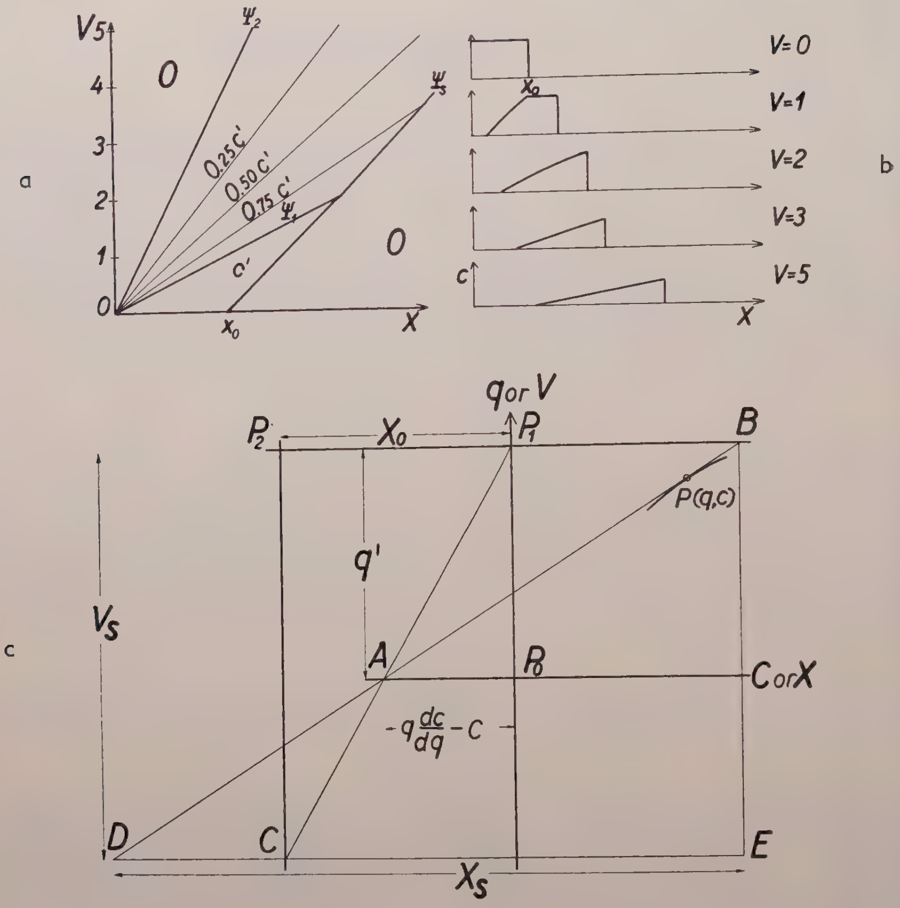
a
V
5
4
3
2
1
0
Ψ_2
0
0.25 c'
0.50 c'
0.75 c'
Ψ_1
Ψ_s
c'
0
x_0
x
b
V=0
V=1
V=2
V=3
V=5
x_0
c
x
c
q or V
P_2
X_0
P_1
B
P(q,c)
q'
V_s
A
P_0
C or X
$-q\frac{dc}{dq}-c$
D
C
E
X_s

Fig. 14. Development of a free band. a) V—x diagram. b) $c(x)$ at various values of V (arbitrary units). c) Geometrical construction of V_s and x_s for a given point (q, c) on the isotherm.

of sorbate in the column will not suffice to build up a front with c varying continuously from 0 to c'; the curve $c(\Psi)$ will as before have $c = 0$ at high Ψ and proceed following (4 b) with decreasing Ψ, but will stop abruptly at a certain concentration c_s because of shortage of sorbate. At c_s, a sharp front is obtained. Let us denote the quantities characteristic of this point by x_s, V_s, Ψ_s, c_s, and q_s, and let us consider the section $V = V_s$ through the solution. In passing through all values of $x = V_s \Psi^{-1}$ from 0 to x_s, it must have passed all the sorbate present, which gives the condition

$$q' x_0 = \int_0^{x_s} q\,dx = V_s \int_0^{\Psi_s} q\,d(\Psi^{-1}) = V_s \int q\,d\left(\frac{dc}{dq}\right) = V_s\left(q\frac{dc}{dq} - c\right)_s. \tag{34}$$

In the last member, all functions have their values at the point (x_s, V_s). Moreover, (4 b) must be valid for this point as for the whole curve;

$$V_s = x_s \left(\frac{d\,q}{d\,c}\right)_s. \tag{35}$$

If $q(c)$ is known, the values of x_s and V_s corresponding to each c_s can be calculated by means of (34) and (35). Fig. 14 c shows a geometrical construction of x_s and V_s.

P is a point (q, c) on the isotherm; P_0 is the origin of coordinates for q and c, $P_0 P_1 = q'$, and $P_1 P_2 = x_0$. The tangent through P cuts the horisontal line $q = 0$ at A, and the horisontal line $q = q'$ at B. A line through P_1 and A cuts the vertical line through P_2 at C, and a horisontal line through C cuts the tangent BPA at D and the vertical line through B at E. In this figure, $P_2C = V_s$, and $DE = x_s$, as can be proved from (34) and (35) bearing in mind that $AP_0 = q \frac{d\,c}{d\,q} - c$. One can also devise other ways of construction, for instance by means of the formula $q' x_0 = q_s x_s - c_s V_s$, which can easily be derived from (34) and (35).

However, the simplest approach is probably in general inserting an approximate equation for $q(c)$ into (34) and (35) and calculating the course of $V_s(x_s)$.

Fig. 14 b shows different stages in the development of a "free band", for the same case as that considered in Fig. 14 a.

I am much indebted to Professor ARNE TISELIUS for the helpful and encouraging criticism he has given in a number of valuable discussions. I should also like to thank Professor GUSTAF LJUNGGREN for his constant and encouraging interest in this work, and Professor ARNE ÖLANDER for giving laboratory facilities for some work in connection with this paper. In the practical work, which has inspired the paper, I have had the pleasure of cooperating with Messrs ERIK EKEDAHL and ERIK HÖGFELDT, who have also read the manuscript, tested its readability, and helped me to make a number of minor improvements.

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SUMMARY

This paper deals with the types of fronts obtained when a solution passes through a sorbent column under such conditions that equilibrium is everywhere obtained between solution and sorbent. The calculations can be much simplified if it is observed that the concentrations c_i and q_i are very often functions of the single variable $\Psi = \frac{V}{x}$ (2), ("the Ψ condition"). The Ψ condition is valid if the sorbent has initially a uniform load, and if the solution entering the column has a constant composition. There are also many other cases where it can be applied after changing the origin of coordinates.

When the Ψ condition is valid, the law of conservation of matter takes the simple forms: $\frac{d\,q_i}{d\,c_i} = \Psi$ (4 b) for broadening (continuous, diffuse) fronts, and $\frac{\triangle\,q_i}{\triangle\,c_i} = \Psi_s$ (5) for sharp fronts. With these two equations the shape of the fronts can be found almost automatically with very little brain work, as is shown by finding the $V-x$ diagrams, and the $c\,(x)$ and $c\,(V)$ curves, for various cases of sorption and desorption of single solutes and mixtures of solutes.

With an ion exchanger it is advantageous to replace Ψ by a quantity $\psi = \frac{a_0\,V}{s_0\,x}$ (15). The corresponding formulae for the fronts are $\frac{d\,\beta_i}{d\,\alpha_i} = \psi$ (17) and $\frac{\triangle\,\beta_i}{\triangle\,\alpha_i} = \psi_s$ (18).

The process of displacement separation can be illustrated in a very condensed way by means of "$l-y$ diagrams", which are discussed in detail for two cases of ion exchange. The $l-y$ diagram shows the minimum length of column required to separate the ions. No better separation is obtained by increasing the length beyond this.

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Institute of Inorganic Chemistry, Chalmers' Institute of Technology, Gothenburg.

Institute of Inorganic and Physical Chemistry, the University, Stockholm.

Research Institute of National Defence, Dept. 1, Ulriksdal.

Notes: Some of the symbols in previous parts differ from those in this paper. In part I, a corresponds to c, and $b_0 - b$ to q. In parts II and III, a/a_0 corresponds to α_2, and s/s_0 to β_2.

The curves in figs. 2, 5, 6, 9, 10, and 14 have been calculated using isotherms of LANGMUIR or (14 a) type.

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On filtration through a sorbent layer

V. "Final form" fronts and broadening fronts

By Lars Gunnar Sillén

With 7 figures in the text

Part IV in this series deals with filtration of a solution through a sorbent layer under such conditions that equilibrium is obtained at every point between solution and sorbent. It was pointed out that the discussion can be greatly simplified by using consistently the variable

$$\Psi = \frac{V}{x}. \tag{1}$$

The free and sorbed concentrations c_i and q_i will be functions of Ψ only ("Ψ condition") provided the sorbent is homogeneous in its initial state ($\Psi = 0$), and the incoming solution ($\Psi = \infty$) always has the same composition. In many other cases (development of bands, displacement analysis) the Ψ condition can be applied after a suitable change of coordinates.

This paper will consider cases where equilibrium is not obtained; then the time enters as a variable, and kinetic equations appear. However, there will always be a strong analogy between the types of fronts formed under non-equilibrium conditions and the curves obtained for equilibrium filtration. Moreover, with increasing x and V, the curves $c\ (\Psi)$ and $q\ (\Psi)$ will approach indefinitely to the equilibrium curves.

In the equilibrium case, one meets with two types of front: sharp fronts with a discontinuous jump in q_i and c_i, and broadening fronts with continuous c_i and q_i. In the non-equilibrium case also, only two types of front are obtained. If we would have got a sharp front in equilibrium, we now find a front that tends towards a *final form* and then moves on through the column with the same speed as the equilibrium front would have had. Otherwise we get *broadening* fronts.

For the particular case of gas sorption, the formation of fronts of final shape is a consequence of the special theoretical assumptions of Mecklenburg (1925), and of Bohart and Adams (1920). Fronts tending to an almost constant shape have also been observed by experimental workers on gas sorption (Weyde and Wicke 1940, Wicke 1939, 1940, Dole and Klotz 1946, Danby *et al.* 1946), reviewed in Parts I and III of this series. Johansson, Persson, and Svensson

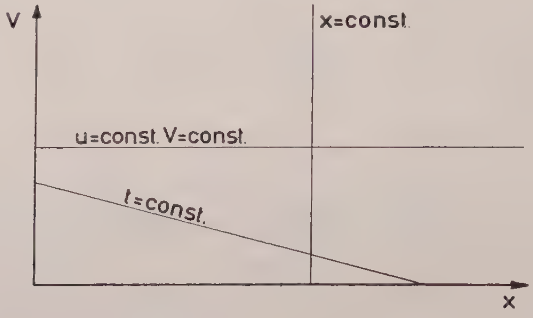


Fig. 1. V—x diagram, showing lines with x = const., t = const., and u (and V) = const.

(1942–1948, p. 20) and SILLÉN (1945, Part I) have shown that fronts of final shape will occur on sorption when the isotherm has the ordinary shape, convex to the q axis.

The theory of final form fronts was applied to the case of ion exchange by EKEDAHL and SILLÉN (1945 and 1947, Parts II and III), who measured down to very low concentrations the leaking curves on displacing H^+ by Ag^+ and found that the curves very quickly seemed to reach a constant form, independent of column length. Later on, GLUECKAUF and COATES (1947) also discussed final forms for special cases of sorption from solutions. It also follows from THOMAS' (1944, 1948) equations (see also HIESTER and VERMEULEN 1948) that final form fronts can be formed in an ion exchanger; this consequence, however, was not stressed by THOMAS, who assumed bimolecular kinetics as did EKEDAHL and SILLÉN.

On the other hand a number of approaches to the theory of sorption columns have appeared in literature, which are based on analogies to heat transfer or to distillation columns, and do not show the important phenomenon of final form fronts. It was thus thought not to be superfluous to give here a general treatment, not confined to any special type of kinetics.

This paper deals also with broadening fronts and discusses, among other things, calculating the sorption isotherm from the form of the fronts and vice versa.

Coordinates and kinetic conditions

In the equilibrium case, the only variables in our equations are x, the length of the column, and V, the amount of solution that has passed through it. Neither the time t sec nor the velocity of flow, v cm sec^{-1} or v_v ml sec^{-1} enter the equations as independent variables. The relation between these variables is:

$$V = uv_v; \qquad u = t - xv^{-1} \tag{2}$$

where u is the "corrected time" employed in Parts I–III of this series. In the processes we are going to consider, the velocity of flow v (and v_v, which is proportional to v) will be assumed to be constant; our equations can then still be formulated so that formally only x and V occur as variables. Fig. 1 shows a V–x diagram with lines u = const. (horisontal line) and t = const. (slanting line).

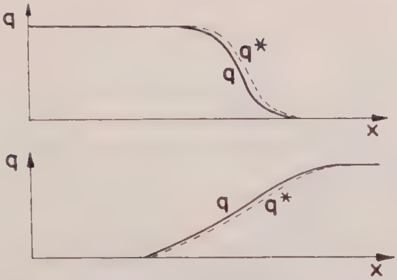


Fig. 2. Non-equilibrium filtration. a) sorption, b) desorption. V = const. Full curve: $q(x)$, dotted curve: $q^*(x)$, the value that would correspond to equilibrium with the prevailing c.

The law of the conservation of matter (IV, 1)

$$\left(\frac{\partial c_i}{\partial x}\right)_V + \left(\frac{\partial q_i}{\partial V}\right)_x = 0 \tag{3}$$

is still valid if diffusion along the column can be neglected. The condition that q_i must correspond at every point to equilibrium with the various c_i at that point is, however, replaced by some kinetic equation

$$\left(\frac{\partial q_j}{\partial t}\right)_x = v_v \left(\frac{\partial q_j}{\partial V}\right)_x = \text{function of (all } q_i \text{, all } c_i). \tag{4}$$

The derivative in (4) also depends on the grain size and other properties of the sorbent, and on the velocity of flow; however, these factors will be assumed to be constant. We shall make no assumption about the function in (4) other than that the process works towards equilibrium: if q_j is less than the equilibrium value q_j^*, the substance A_j is sorbed (q_j increases); if $q_j > q_j^*$, it decreases, thus A_j is desorbed.

If, for a given V, we draw the curves $q_i\ (x)$ and $q_i^*\ (x)$, the q_i curve will always be found to lag behind, either the substance is being sorbed or desorbed (Fig. 2). Or, one can say that the q curve is shifted to higher Ψ values as compared with the q^* curve. If, however, q and q^* are plotted as functions of Ψ for a series of increasing V, a certain increment in Ψ, $\delta\Psi$, will correspond to a longer and longer time. For this reason, the two curves $q(\Psi)$ and $q^*(\Psi)$ will approach indefinitely to one another; in the limiting case where they coincide, the curve $q(\Psi)$ will be the same as that obtained in the equilibrium case.

We conclude that with increasing V and x, the functions $q_i(\Psi)$ *and* $c_i(\Psi)$ *will approach indefinitely to the corresponding curves for equilibrium.*

Final form fronts

An especially simple case is met with in the cases where one would have got a sharp front in equilibrium. The front will then approach to a final form which moves indefinitely with constant velocity through the sorbent column.

This is equivalent to saying that for large x and V, c_i and q_i become functions of the single variable

$$z = x - w\,u \tag{5}$$

where w is the velocity of the front in cm sec^{-1}, using the corrected time u.

We introduce the quantity

$$\Psi_s = v_v\, w^{-1}. \tag{6}$$

To begin with, we make no other assumption about Ψ_s than that it is constant; we shall see below that it is identical with the Ψ value found for the sharp front at equilibrium. From (2), (5), and (6) we find

$$z = x - \Psi_s^{-1}\, V. \tag{7}$$

We shall consider first single "final form fronts", and then groups of fronts moving with the same velocity, such as are met with in displacement analysis. In other cases, such as frontal analysis, the fronts move with different velocities, so that the distances between them are steadily increasing, and the fronts can finally be treated as separate single fronts.

Single front. General case

A front of constant form is moving through the sorption column. Far ahead of the front ($z \to +\infty$), c_i' and q_i' are valid for the substance A_i; far behind the front ($z \to -\infty$), the quantities are c_i'' and q_i''. By means of (7) we transform the partial derivatives of (3) to complete derivatives in z and find:

$$\frac{d\,c_i}{d\,z} - \Psi_s^{-1}\frac{d\,q_i}{d\,z} = 0$$

and thus

$$q_i = \Psi_s c_i + (\mathrm{const})_i. \tag{8}$$

This is the equation of a straight line, which must pass through the two points q_i'', c_i'', and q_i', c_i'. From this condition we find

$$\Psi_s = \frac{q_i'' - q_i'}{c_i'' - c_i'} \tag{9}$$

$$(q_i - q_i') = \Psi_s\,(c_i - c_i') \quad \text{or} \quad \frac{q_i - q_i'}{q_i'' - q_i'} = \frac{c_i - c_i'}{c_i'' - c_i'}. \tag{10}$$

From equation (9), the constant Ψ_s is identical with the Ψ_s value for the sharp front one would have obtained in the equilibrium case (IV, 5). In (7), $\Psi_s^{-1}\,V$ is thus the x coordinate that the sharp front would have had in the equilibrium case, and z is the distance from that "equilibrium position".

Fig. 3 shows a q–c diagram, when only one solute A_i is present; the curve is the isotherm for equilibrium, $q^*(c)$. It follows from formulae (8–10) that all sets (q_i, c_i) must fall on the straight line between the two extreme points (q_i'', c_i''), and (q_i', c_i'), which are both on the isotherm. If the isotherm, like that

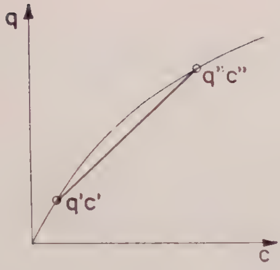


Fig. 3.

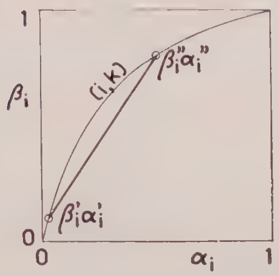


Fig. 4.

Fig. 3. Non-equilibrium sorption of single solute. Initial state: $q' c'$, final state $q'' c''$. Curve: isotherm $q^* (c)$. Thick line: Equation (10), $q (c)$ in the final form of the front. Fig. 4. Non-equilibrium displacement of ion k with ion i on an ion exchanger. Initial state β_i', α_i', final state β_i'', α_i''. Curve: isotherm (i, k). Thick line: Equation (17), $\beta (\alpha)$ in the final form of the front.

in Fig. 3, is of the ordinary, convex type, $q < q^*$ for all intermediate points, which means that *sorption* must take place in the front. A process with a front of final form is thus possible only for the case of sorption ($q_i'' > q_i'$), and not for desorption ($q_i'' < q_i'$) (Part I, p. 18). This is also the condition for a sharp front in equilibrium sorption.

If there is initially no A_i in the column ($c_i' = q_i' = 0$), (10) takes the form

$$q_i = \Psi_s c_i = \frac{q_i''}{c_i''} c_i. \tag{11}$$

Ion exchange

For an ion exchanger, the law of conservation of matter takes the form (IV, 13 b; the symbols are the same as in Part IV)

$$\frac{\partial \alpha_i}{\partial (s_0 x)} + \frac{\partial \beta_i}{\partial (a_0 V)} = 0. \tag{12}$$

We introduce the quantity ψ_s (cf. 6; w cm sec^{-1} = velocity of front)

$$\psi_s = \frac{a_0 v_v}{s_0 w}. \tag{13}$$

The condition that α_i and β_i be functions of the variable z only, where

$$s_0 z = s_0 x - a_0 V \psi_s^{-1} \tag{14}$$

gives, together with (12), the condition

$$\beta_i = \psi_s \alpha_i + (\text{const.})_i. \tag{15}$$

This again is the formula of a straight line, which must pass through the limiting values, (β_i', α_i') for large positive z, and (β_i'', α_i'') for large negative z. We find

$$\psi_s = \frac{\beta_i'' - \beta_i'}{\alpha_i'' - \alpha_i'} \tag{16}$$

$$\beta_i - \beta_i' = \psi_s (\alpha_i - \alpha_i') \quad \text{or} \quad \frac{\beta_i - \beta_i'}{\beta_i'' - \beta_i'} = \frac{\alpha_i - \alpha_i'}{\alpha_i'' - \alpha_i'}. \tag{17}$$

Here, again, the constant ψ_s is identical with the ψ_s value for the sharp front at equilibrium. Fig. 4 shows the isotherm (i, k), that is the curve $\beta_i(\alpha_i)$ in the case where only two types of cations, A_i^+ and A_k^+ are present. A_i^+ is assumed to be more strongly sorbed than A_k^+. All points on the line (17) are below the equilibrium curve, which means that A_i^+ is being sorbed in the front, which is possible only if $\beta_i'' > \beta_i'$. A *final form front* is thus possible *only if the ion added is more strongly sorbed* than the old one. Otherwise a broadening front is obtained.

If for any ion, $\alpha_i' = \beta_i' = 0$, then for this ion

$$\beta_i = \psi_s \alpha_i = \frac{\beta_i''}{\alpha_i''} \alpha_i. \tag{18}$$

If, moreover, for the same ion $\alpha_i'' = \beta_i'' = 1$, then $\psi_s = 1$, and for this ion

$$\beta_i = \alpha_i. \tag{19}$$

Final forms for LANGMUIR kinetics

The exact form of the final functions $q(z)$ and $c(z)$ depends on the kinetic conditions (4). We shall calculate them for the simple assumption of LANGMUIR kinetics.

For the general case of sorbent, with only one solute present, this takes the form

$$-\frac{\partial q}{\partial V} = \frac{\partial c}{\partial x} = -kc(q_0 - q) + k_1 q. \tag{20}$$

Here, k and k_1 are constants, and q_0 is the maximum load of the sorbent. This assumption will lead to a LANGMUIR isotherm:

$$k_1 q'' = kc''(q_0 - q'') \tag{21}$$

since the point q'', c'' must be on the isotherm.

If $q' = c' = 0$, (11) must be valid for the final state:

$$q = c\Psi_s = \frac{q''}{c''} c. \tag{22}$$

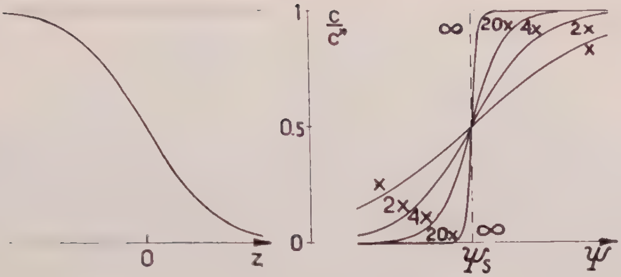


Fig. 5. Final form of sorption front with Langmuir kinetics, equation (23). a) $\frac{c}{c''}$ as a function of z. b) $\frac{c}{c''}$ as a function of Ψ for increasing x (x, $2x$, $4x$, $20x$, ∞). Note that the curves approach to a vertical line (the equilibrium position of the front).

Introducing (7), (21), and (22) into (20), we find

$$\frac{dc}{dz} = -k\Psi_s c\,(c'' - c)$$

which gives the solution

$$\frac{q}{q''} = \frac{c}{c''} = \frac{1}{1+Z};\ Z = e^{kq''z}. \tag{23}$$

This equation is almost identical with (I, 35); the main difference is that in (23) the velocity of flow v enters the constant k, which is inessential, since k is at any rate a function of v. Fig. 5 shows $c(z)$, and $c(\Psi)$ for several x. GLUECKAUF and COATES (1947) have later on calculated a final form assuming a LANGMUIR isotherm but not LANGMUIR kinetics. Their result (eq. 11 in their paper) is more complicated than and not identical with (23).

We now consider an *ion exchanger*, which is originally saturated with A_1^+, and through which a solution of A_2^+ is passed; the latter ion being more strongly sorbed than the former. The letters α and β refer to A_2^+. Our initial conditions are thus: $\alpha' = \beta' = 0$, $\alpha'' = \beta'' = 1$.

With bimolecular kinetics we have, regardless of the boundary conditions,

$$-\frac{\partial\beta}{\partial(a_0 V)} = \frac{\partial\alpha}{\partial(s_0 x)} = -k\alpha(1-\beta) + k_1\beta(1-\alpha). \tag{24}$$

In the final state, from (19)

$$\psi_s = 1,\ \alpha = \beta$$

and from (14)

$$s_0 z = s_0 x - a_0 V$$

which gives

$$\frac{d\alpha}{d(s_0 z)} = -(k - k_1)\ \alpha(1-\alpha). \tag{25}$$

The solution is

$$\beta = \alpha = \frac{1}{1+Z}; \quad Z = e^{(k-k_1)s_0 z}. \tag{26}$$

This formula is essentially identical with formulae (II, 9) and (III, 4) in previous papers of EKEDAHL and SILLÉN (Parts II and III), but for that v has been included in k and k_1. We found experimentally that this formula, which contains only one arbitrary constant, gives a surprisingly good description of the data in the case studied (displacement of H^+ by Ag^+).

Displacement separation

In Part IV we showed that if a number of ions 1, 2, 3 ... are displaced along an ion exchanger column by an ion, N, which is more strongly sorbed than any of them, and if these ions displace another ion, O, which is less strongly sorbed, then the ions will finally separate completely into regions arranged in the order of sorbability. In the non-equilibrium case, the final state is much the same, only that the fronts are not sharp, but have aquired an other, constant form, which need not be the same for all the different fronts. However, they travel with the same velocity (Fig. 6). For the final functions, (15) must be valid:

$$\beta_i = \psi_s \alpha_i + (\text{const.})_i .$$

Now, $\alpha_N = \beta_N = 0$ for high positive z. If also $\alpha_N = \beta_N = 1$ for large negative z, then $\psi_s = 1$, and for all ionic species $(\text{const.})_i = 0$ and

$$\beta_i = \alpha_i \tag{27}$$

since $\alpha_i = \beta_i = 0$ for some z values.

If the ion A_0^+ (which may be H^+) is also present in the displacing solution, β''_N and α''_N are not equal to 1, and we find

$$(i \neq 0) \; \frac{\beta_i}{\alpha_i} = \psi_s = \frac{\beta''_N}{\alpha''_N} = \frac{\beta_0 - \beta''_0}{\alpha_0 - \alpha''_0}. \tag{28}$$

For the general case we find, if we use a pure substance for the displacement

$$\frac{q_i}{c_i} = \Psi_s = \frac{q''_N}{c''_N} \tag{29}$$

which must be true at all points and for all substances. This is TISELIUS' equation (1943), which is thus still valid for non-equilibrium displacement.

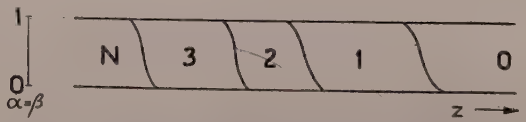


Fig. 6. Final form of the fronts on separation of ions by displacement.

Broadening fronts

Isotherms from equilibrium elution curves

Measurements on broadening fronts at low velocities of flow, thus approximate equilibrium, can be used for calculating sorption isotherms (DE VAULT 1943). In principle, one single experiment is needed. A sorption column of length x is initially loaded with the sorbate A at a concentration q_0, expressed for instance in mmol cm. From $V = 0$ onwards, a current of pure solvent is introduced at the inlet end of the column, and the concentration c in the solution leaving it at the other end is measured as a function of V. Since the Ψ condition is valid, we find from (IV,4 b)

$$\frac{dq}{dc} = \Psi = \frac{V}{x} \tag{30}$$

from which follows (compare Fig. 7 a)

$$\frac{1}{x}\int_{c}^{c_0} V\,dc_i = \int_{c}^{c_0} \Psi\,dc = q_0 - q$$

or

$$q\,x = q_0\,x - \int_{c}^{c_0} V\,dc. \tag{31}$$

In (31), q is valid at the very end of the column, in equilibrium with c; with the aid of this formula we can thus calculate the isotherm $q(c)$.

We notice that in (31) q and x do not occur separately but only as products: $q_0\,x$, the amount originally sorbed, and $q\,x$, the amount the column would have contained had the load been the same throughout the column as at its end. It is thus not essential that x be expressed in cm, and q in mmol/cm or g/cm. We can let x be any quantity that is proportional to the amount of sorbent, such as the volume, or weight, or maximum capacity of the sorbent, as long as we express q in the corresponding units.

What is measured is generally not V but a quantity

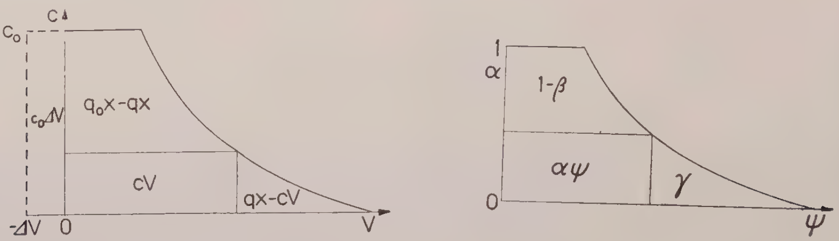
$$V' = V + \varDelta V \tag{32}$$

where the quantity $\varDelta V$, which contains the "hold-up" of the column, is not always accurately known. If the measurements are extended practically to $c \approx 0$, we can calculate

$$\int_{0}^{c_0} V'\,dc = \int_{0}^{c_0} V\,dc + c_0\,\varDelta V = q_0\,x + c_0\,\varDelta V. \tag{33}$$

With (33) we can find whichever of $q_0\,x$ and $\varDelta V$ is less accurately known.

Fig. 7 a shows the areas corresponding to the quantities of sorbate that have been eluted and that are still left in the column:



7 a

7 b

Fig. 7. Desorption (elution) of a single solute. a) $c-V$ diagram (elution curve) for the general case. b) α-ψ diagram for an ion exchanger. On each domain its area has been marked out.

Quantity eluted:
$$\int_0^V c\,dV = cV + \int_c^{c_0} V\,dc = cV + q_0x - qx \tag{34}$$

left in column:
$$q_0x - \int_0^V c\,dV = q_0x - cV - \int_c^{c_0} V\,dc = qx - cV. \tag{35}$$

The equilibrium of two ions, say A_1^+ and A_2^+, on an ion exchanger can be studied in an analogous way. The sorbent column is initially saturated with the ion that is more strongly held (say, A_2^+), and a current of a solution with A_1^+ as its only cation is passed through the column, while the fraction of A_2^+ is measured in the effluent solution. The letters β and α will refer to the ion A_2^-.

The Ψ condition is valid, and (IV, 17 b), $\frac{d\beta}{d\alpha} = \psi$, gives

$$\frac{1}{s_0x}\int_\alpha^1 a_0 V\,d\alpha = \int_\alpha^1 \psi\,d\alpha = 1-\beta$$

$$\beta s_0x = s_0x - a_0\int_\alpha^1 V\,d\alpha \tag{36}$$

$$\beta = 1 - \int_\alpha^1 \psi\,d\alpha. \tag{37}$$

Here, β is the fraction of A_2^+ sorbed at the very end of the sorbent, and thus in equilibrium with α. Just as before, ΔV or s_0x can be obtained from

$$a_0\int_0^1 V'\,d\alpha = s_0x + a_0\,\Delta V. \tag{38}$$

If the amount of A_2^+ that is left in the ion exchanger, is denoted by γs_0x, the amount eluted is

$$(1-\gamma)\,s_0x = \int_0^V a_0\,\alpha\,dV = a_0V\alpha + \int_\alpha^1 a_0V\,d\alpha$$

or, dividing by s_0x

$$1 - \gamma = \alpha \psi + \int_{\alpha}^{1} \psi \, d\alpha = \alpha \psi + 1 - \beta$$

from which

$$\gamma = \beta - \alpha \psi. \tag{39}$$

In the next paper (Part VI) it will be shown that isotherms $\beta(\alpha)$ obtained by means of (37) from a single elution experiment agree very well in a wide concentration range with the points obtained by the much more laborious way over static equilibrium measurements.

Choice of graphic representation

If the isotherm $q(c)$ or $\beta(\alpha)$ is known with sufficient accuracy, it is possible on the other hand to calculate the course of an elution curve at equilibrium, $c(\Psi)$ or $\alpha(\psi)$, curves to which the non-equilibrium curves approach for small velocities of flow or for large V and x. To be specific, let us consider an ion exchanger and ask what sort of representation of the equilibrium data is best. A table or plot of β as a function of α is not very sensitive, unless a large scale is used. Plots of log α, log β, or (log α — log $(1 - \alpha)$) as functions of ψ have the disadvantage of being infinite in one or two directions. A more suitable function, which gives both good accuracy and a condensed plot, would perhaps be the equilibrium quotient $\varkappa$

$$\varkappa = \frac{\beta(1-\alpha)}{(1-\beta)\alpha} = \frac{\alpha^{-1}-1}{\beta^{-1}-1}. \tag{40}$$

If we assume that $\varkappa$ and $\varkappa' = \dfrac{d\varkappa}{d\beta}$ are given as functions of β, we can easily calculate α and ψ

$$\alpha = \beta[\beta + \varkappa(1-\beta)]^{-1}; \quad \psi = \frac{[\beta + \varkappa(1-\beta)]^2}{\varkappa - \varkappa' \beta(1-\beta)}. \tag{41}$$

If $\varkappa$ is really constant $= K$, i.e. if an ideal isotherm is followed,

$$\psi = [\beta + K(1-\beta)]^2 K^{-1}. \tag{42}$$

If, for a non-equilibrium elution experiment, the values of $\alpha(\psi)$ are inserted into (37), we find a value of β, β_f, which is not indentical with the β in the sorbent. If this false β_f is introduced into (40) together with the corresponding α, we get a function $\varkappa_f$, which is certainly a false equilibrium constant since it is neither constant nor refers to an equilibrium. On the other hand, a plot of $\varkappa_f(\beta_f)$ is a very clear and compact way of representing the experimental data for a broadening front, and curves of this type may prove suitable for comparing the results with various length of column, velocities of flow, etc., and perhaps also for predicting the performance of columns under various conditions. The value of this proposal can only be judged after a not too small number of such curves has been prepared under various conditions.

For the general case of sorption it may perhaps sometimes also prove advantageous to use some plot that is more sensitive than $q(c)$. One might assume formally a LANGMUIR isotherm, with one constant (say q_0) really constant, and the other variable, and plot this second "constant" as a function of q or c.

Analogies previously attempted

A comparatively large amount of work has been spent calculating theoretical sorption and desorption curves for the case of a linear isotherm, $\alpha = \beta$ ($K = 1$) for an ion exchanger, $q = kc$ in the general case. One reason for this may be that for a linear isotherm the partial differential equations obtained from plausible assumptions on the kinetics can generally be solved exactly by classical methods, and that analogies from the theory of heat transfer could be used. (JOHANSSON, PERSSON and SVENSSON 1942–48, BOYD, MYERS and ADAMSON 1947, HOUGEN and MARSHALL 1947, and many others.)

One may ask whether there are really any cases in nature where an exactly linear isotherm is obtained. Two cases which may come rather close to this ideal may be the exchange of two isotopic ions on an ion exchanger, and partition chromatography, in so far as a constant partition coefficient can be assumed. In neither of these cases is the linearity complete because of the small difference in equilibrium constant for various isotopes and because of the deviations from the ideal partition laws. Nevertheless, a linear isotherm has proved a useful approximation for many cases of partition chromatography.

For sorption processes, however, this focussing of the attention on cases with linear isotherms seems unfortunate, since a linear isotherm is a singular case, which would give fronts showing the characteristics of neither of the two immensely more important groups of fronts, the final form fronts, and the broadening fronts. To start with the equilibrium case, with a linear isotherm one would certainly get sharp fronts (at $\psi = 1$, or $\Psi = k$) as long as the Ψ condition is valid. However, these fronts would be sensitive to small disturbances, since they would not be able to sharpen themselves; under equilibrium conditions, a front of any shape would travel unchanged through the column. Under non-equilibrium conditions, a front of final form cannot develop; if it could, all the (q, c) points in Fig. 3 would fall on the equilibrium line (the isotherm), and no sorption would take place. Thus the front broadens, but not as much as the normal type of broadening fronts. If for one of these latter fronts, q or c is plotted as a function of Ψ, the points will approach a continuous curve (the equilibrium curve) with increasing V and x, whereas for a linear isotherm the points $c(\Psi)$ or $q(\Psi)$ will nevertheless finally approximate to a vertical line.

The results calculated for a linear isotherm will thus disagree with what is observed in most experimental sorption or desorption runs. The application of such theories to experimental data has for this reason generally met with disappointing results; for instance, BOYD, MYERS, and ADAMSON (1947), report that "the unexpected variation of the rate constant, k, with bed depth suggests there are factors involved not considered by the theory".

Another analogy, which has been attempted since a well-developed theory could be taken over, is the analogy with distillation columns. There are however fundamental differences between a sorption column and a distillation column.

If the separation is achieved by displacement, a good separation with sharp fronts or (for non-equilibrium) final form fronts is obtained. The separation can be improved by decreasing the rate of flow. But (and here comes one difference) nothing can be gained by increasing the length of the column very much over the minimum length required for a complete separation (Part IV). At equilibrium, the separation is at any rate complete; otherwise the "tails" of the various regions will retain their form, however long the column is made. — For moderate rates of flow, these tails cause by the way much less trouble than bulges on the fronts, meaning that the surfaces Ψ = const. are no longer planar; it seems to be rather difficult to get rid of them, even with careful packing of the sorbent (cf. CLAESSON 1946 a). However, HAGDAHL (1948) and CLAESSON (1946 b) have shown ingenious ways of minimizing their influence and still keeping a high column capacity.

As in the previous paper, I should like to thank Professors GUSTAF LJUNGGREN, ARNE TISELIUS, and ARNE ÖLANDER, and Messrs ERIK EKEDAHL and ERIK HÖGFELDT for valuable discussions and for helping me in many other ways in connection with this paper.

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Institute of Inorganic Chemistry, Chalmers' Institute of Technology, Gothenburg.
Institute of Physical and Inorganic Chemistry, The University, Stockholm.
Research Institute of National Defence, Dept. 1, Ulriksdal.

SUMMARY

Non-equilibrium processes in sorption columns are best treated by first considering the corresponding equilibrium process. With increasing V and x the curves $c_i(\Psi)$ and $q_i(\Psi)$ will approach indefinitely to the equilibrium curves ($\Psi = \frac{V}{x}$ as in part IV).

Where a sharp front would have been formed at equilibrium, a *final form front* is obtained for non-equilibrium. The front finally moves with constant form and constant velocity. Then a linear relation exists between q and c, and they are functions of the single variable $z = x - \Psi_s^{-1} V$, which is the distance from the position the sharp front would have had at equilibrium.

The final form has been calculated for two special cases: LANGMUIR kinetics, and bimolecular ion exchange kinetics; the results, (23) and (26), coincide with those in parts I–III. However, the treatment in this paper is otherwise not confined to any special type of kinetics.

TISELIUS' equation for displacement processes is valid for non-equilibrium, too.

The other type of front is the *broadening front;* its form is governed by the condition that $c_i(\Psi)$ and $q_i(\Psi)$ approach to the curve for equilibrium. The use of elution curves for calculating the isotherm and vice versa is discussed.

The elution curve $\alpha(V)$ of an ion exchanger can, if equilibrium is not obtained, be used for calculating false values β_f and $\varkappa_f$ for β and the equilibrium quotient. Curves $\varkappa_f(\beta_f)$ may prove a suitable condensed way of representing

measurements of non-equilibrium elution curves. — A similar treatment might also be applied to other types of sorbent as well.

Several theoretical treatments of sorption processes in columns are based on assumptions, such as formal analogy to heat transfer, which lead to a linear isotherm. A linear isotherm has proved a useful approximation in many cases of partition chromatography. For sorption processes in general, the focussing of the attention on linear isotherms seems unfortunate, since this is a singular case giving fronts which have the characteristics of neither of the two much more important types of sorption front, final form fronts and broadening fronts.

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Serum fibrinolysin activation in the experimental hypersensitivity of the rabbit. II. Trypsin inhibitor activity

By Hans v. Euler and Leo Heller

In the first paper of this series (1) we reported the results of a few experiments pertaining to the question of the proteolytic activity of the serum in the experimental hypersensitivity of rabbits, produced by intravenous injections of massive doses of horse serum, a condition considered as one of the best so far available models of the rheumatic allergy of man (2, 3). (For a critical review of this point compare reference (4)). It falls beyond the scope of the work to be reported in the following to try to evaluate the degree of identity between both of the conditions named, but the rheumatic disease is still considered by the majority of experts as a peculiar kind of anaphylactoid hypersensitivity and the results obtained in other kinds of anaphylactic or anaphylactoid reactions may have some bearing on our understanding of the pathogenesis of rheumatism. In our case, the experiments performed with the aim of demonstrating an actual or potential activation of serum proteolysis, as postulated by several authors (5, 6, 7), eventually occurring during the development of hypersensitivity, failed to yield any support to the above hypothesis.

The method of sensitization of the rabbits was the same as described by Rich and Gregory (2) and has been already used by us in earlier work (1). Both rabbits injected with massive doses of horse serum developed a condition of hypersensitivity against the injected antigen, as demonstrated by the strongly positive result of skin tests, performed 3 weeks after serum injections. That the hypersensitivity was not limited to the skin could be shown by a positive result of intraarticular injection of the antigen into the cavity of the knee joint performed at the same time (3). Control injections of saline into symmetrical joints or of the same antigen into the joints of unsensitized rabbits (2 animals) gave no demonstrable reactions while the injection of the same antigen into the joints of both sensitized animals resulted in a distinct infiltration and oedema, easily palpable through the skin.

The viscosimetric technique for measuring proteolysis (8), however, used by us previously (1), was now abandoned because of its apparently too low sensitivity, its high inherent error range (9) and its unfittedness for serial comparative determinations. It was substituted by the fibrinogenolytic technique of Ferguson (10), as recommended by Ungar and Mist (6), which in our opinion proved to be superior to viscosimetry both in regard to the sensitivity, to the duplicability of its results and to the ease of manipulation. Also the way of preparing the euglobulin solutions for fibrinolysin determinations was changed in the course of the present investigation.

Instead of using the method of isoelectric precipitation of euglobulin from diluted serum, which was found to result in partial denaturation and insolubility of the precipitate unless performed in the cold under exactly controlled conditions, we followed the example of CHRISTENSEN et al. (8), who salts out the euglobulin fraction at 33 per cent saturation of ammonium sulphate, obtaining an almost perfectly soluble product. The ammonium sulphate precipitated serum euglobulin was routinely dissolved in phosphate-buffered saline (6) (pH = 7.4) containing sodium merthiolate 1: 10 000 to eliminate bacterial growth, the quantity of the buffer used being 0.4 ml per 1.0 ml serum. 0.2 ml volumes of these euglobulin preparations were then diluted serially (1: 2) in a row of small agglutination tubes. A lyophilized preparation of purified streptokinase, dissolved in phosphate-buffered saline, was used as activator. A volume of 0.1 ml of liquid, containing 10 international units of streptokinase, corresponding to a slight excess of the activator when tried on a preparation of normal human euglobulin, was added to each agglutination tube together with 0.2 ml of a 0.2 per cent solution of bovine plasma fibrinogen (Fraction I), supplied by Armour Laboratories. dissolved in the same buffer. The racks were incubated one hour at 37° C. Finally, one drop of thrombin solution (HOFFMAN–LA ROCHE), containing a low multiple (5—10) of the minimal dose needed to cause instantaneous coagulation of fibrinogen in euglobulin-free controls, was added to all the tubes. The presence or absence of gelation in all tubes was then noted and the proteolytic activity of the serum tested expressed as the reciprocal of the highest euglobulin dilution giving no gel after addition of thrombin (the 1st tube containing undiluted euglobulin).

When testing our rabbit sera, a preparation of normal human euglobulin was always used as control, yielding activities oscillating between the relatively narrow limits of 128—512 (average value 256). As additional control, a tube containing only fibrinogen and activator plus buffer instead for euglobulin dilution was treated in the same way as the other tubes. The above high fibronogenolytic activity constantly shown by several normal human sera after streptokinase activation (unactivated human euglobulin was found to be practically devoid of any fibrinogenolytic action unless allowed to age at least 2—3 days, thus perfectly confirming the earlier findings of other workers) should allow us to discover any significant activity of our rabbit euglobulin preparations down to c:a 1 per cent of the activity of human serum. So far, only the streptokinase kind of activation has been tested by us by the fibrinogenolytic method, using freshly precipitated euglobulin preparations. No significative activity could be found for sera from both normal and hypersensitized rabbits in the above experiments (compare Table I). That negative result should not, however, be interpreted as a proof of the absence of profibrinolysin in rabbit sera (c. f. TILLETT and GARNER (11)), for it has recently been shown by GERHEIM and FERGUSON (12) that rabbit plasma (as well as certain other animal plasmas) does contain varying quantities of a proenzyme activable by staphylococcal but not by streptococcal kinase. Our earlier experiments and the above very recent communication are so far the only ones reporting successful fibrinolysis by rabbit plasma enzyme. LACK (13) failed consistently to activate rabbit plasma with his staphylokinase preparation. The question of the presence

Table I.

Determinations of Fibrinolysin Activity in Sera of Sensitized and Unsensitized Rabbits.

Rabbit N:o	Serum Sample N:o	Time After Horse Serum Injection	Number of Streptokinase Units Added	Fibrinolysin Titer	Remarks
4	1	0 (Unsensitized)	20	2	
	1	»	200	0	
	1	»	20	0	Euglobulin 3 days old
	2	»	10	1	
	2	»	10	0	
	2	»	10	1	
	2	»	—	0	Horse serum added (compare Ref. 1)
5	1	»	10	1	
	1	»	10	0	
	2	7	10	0	
	2	»	10	1	
	3	14	10	1	
6	1	0 (Unsensitized)	10	0	
	2	14	10	0	
	2	»	10	0	
	2	»	10	0	Horse serum added
	3	21	10	0	
	3	»	10	0	
Control human serum:					
2BH		Isoelectric precipitation of euglobulin	20	256	
			20	50	
			0	0	
3ÅL		Ammonium sulphate precipitation of euglobulin	10	128	
			5	256	
			10	512	

of a proteolytic proenzyme in rabbit plasma or serum cannot thus be considered as definitely solved. In the light of the conflicting evidence pertaining to that point (compare also our previous paper (1)), the possibility of an unusually high inhibitor content of the rabbit serum, put forward by LACK (13), must be seriously considered.

The inhibitor problem is at the same time intimately connected with the hypotheses which stress the rôle of proteolytic processes in the anaphylactic (5, 6) or rheumatic (7) types of hypersensitivity, which hade constituted the point of issue for the present series of investigations. According to the hypothesis of MIRSKY (7), a person susceptible to rheumatism should be one not adequately inhibiting the proteinases activated by streptococcal or other kinases. However, in the case of human rheumatic fever patients, no decrease in the trypsin inhibitor activity of the serum during the course of the infection could be reported by TODD (14). On the contrary, the serum inhibitor curves of a group of scarlet fever patients who developed rheumatic fever showed a distinct, though relatively slight increase at the time of the rheumatic activity.

Table II.

Trypsin Inhibitor Activity of Sera from Sensitized and Unsensitized Rabbits.

Rabbit N:o	Serum Sample N:o	Time After Horse Serum Injection days	Number of Trypsin Units Used	Trypsin Inhibitor Titer
4	1	0 (Unsensitized)	8	64
		»	8	32
		»	8	128
		»	8	128
5	1	0	8	128
		»	8	128
		»	8	256
	3	14 days	8	128
6	2	14 days	8	256
		»	8	128
		»	8	256
	3	21 days	8	256
Control human serum:				
3ÅL			8	128
			8	32(?)
			8	128
			8	128
			8	128

The trypsin inhibitor titrations were performed using the same fibrinogenolytic technique as for fibrinolysin determinations, only substituting constant amounts of previously titrated trypsin (MERCK) solution for serial dilutions of euglobulin. Serial twofold dilutions of the tested sera were then added to the tubes containing the enzyme (instead of streptokinase, but in the same volume of 0.1 ml). The addition of the substrate (fibrinogen), the incubation, the challenge by thrombin and the readings were performed as in fibrinolysin activity determinations (see above). Trypsin inhibitor titers of the sera were correspondingly expressed as reciprocals of the highest serum dilution inhibiting the cleavage of fibrinogen by the added constant amount of trypsin. The quantity of the enzyme solution to be used was determined in a preliminary titration in which a 1 % solution of trypsin in phosphate-saline was serially diluted and tested with fibrinogen and thrombin as described above. The last tube giving no gel after addition of thrombin was considered to contain 1 trypsin unit. For inhibitor titrations, 10 such units were used.

In our experiments, as shown by the Table II, no significative changes in the trypsin inhibitor activity of the rabbit serum after hypersensitization against horse serum could be observed. The inhibitor titer of all the tested rabbit sera was approximately of the same order as that of the human control sera (Table II). It is therefore highly unprobable that the difficulties of obtaining proteolysis with rabbit serum or its euglobulin fraction could be accounted for by an unusually high inhibitor level in the blood of that animal. According to our earlier results (1) and to those of Gerheim and Ferguson (12), it seems possible to activate the rabbit plasma resp. serum protease under certain, at present not yet very well understood conditions. Further activation experiments, using staphylokinase preparations, are being performed in the hope of helping to elucidate the problem of the existence of a fibrinolysin precursor in the serum of normal and hypersensitized rabbits. The solution of that problem seems rather important for the ultimate evaluation of the rôle of serum proteolysis in the anaphylactic (and rheumatic) type of hyperergy and for our understanding of its pathogenetical mechanism.

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Mixed oxides with layer lattices

III. Structure of $BaBi_4Ti_4O_{15}$

By Bengt Aurivillius

With 4 figures in the text

X ray studies on the compounds $CaBi_2Nb_2O_9$ (1) and $Bi_4Ti_3O_{12}$ (2) have shown that the comparatively complicated chemical formulae of these compounds can be explained by simple layer structures being built up from $Bi_2O_2^{2+}$ layers and perowskite layers. The unit cells are pictured schematically in Figs. 1 a and 1 c. It was found both for $CaBi_2Nb_2O_9$ and $Bi_4Ti_3O_{12}$ that the symmetry was body-centered pseudo-tetragonal and that the length of the *a* axes had the same value (3.8 Å) while the length of the *c*-axis was 25 Å for $CaBi_2Nb_2O_9$ and 33 Å for $Bi_4Ti_3O_{12}$. In both structures the heavy atoms form approximately a "substructure" with a smaller body-centered tetragonal cell with $a = 3.8$ Å and $c = 25.5$ Å for $CaBi_2Nb_2O_9$ or $c = 33/7$ Å for $Bi_4Ti_3O_{12}$.

The Bi_2O_2 layers and perowskite layers lie perpendicular to the *c*-axis. Similar layer structures have been found for a number of bismuth oxicompounds (3,4,5). The common structural element in all these compounds is quadratic Bi_2O_2 layers between which halides or certain radicals are inserted. This explains the fact that the *a* axes of all these compounds are of about the same length. For a survey see (5).

For the $CaBi_2Nb_2O_9$ type each perowskite layer has the composition $(CaNb_2O_7)_n$ and the height of the layer is equal to four distances Nb—O or approximately to the height of two $E2_1$ (perowskite) unit cells (see Fig. 1 a). A compound with a somewhat similar structure has previously been investigated by Lagercrantz and Sillén (5). In this structure (see Fig. 1 b), beyerite $CaBi_2O_2(CO_3)_2$, the point positions corresponding to the positions of the Nb atoms in $CaBi_2Nb_2O_9$ are occupied by "rotating" CO_3^{2-} groups.

For the $Bi_4Ti_3O_{12}$ type the perowskite layers have the composition $(Bi_2Ti_3O_{10})_n$ and the height of the layer is equal to six distances Ti—O or approximately to the height of three $E2_1$ unit cells.

The general formula for a compound built up in a way similar to $CaBi_2Nb_2O_9$ but where the height of the perowskite layer enclosed between a pair of Me_2O_2 layers is equal to the height of *m* $E2_1$ cells, will be:

$Me_2O_2(Me'_{m-1}R_mO_{3m+1})$. Me, Me′: Ca, Sr, Ba Bi (K + Bi)/2 etc.

R: Ti, Nb, Ta (Nb + Ti)/2 etc.

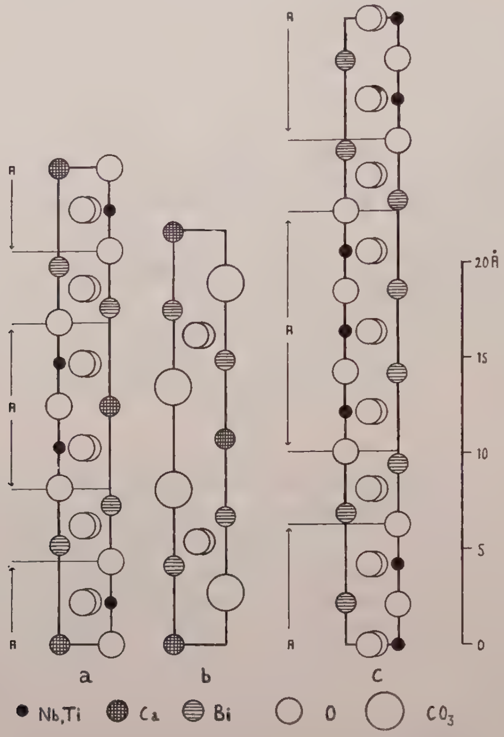

Fig. 1. Schematical pictures of the structures af a. $CaBi_2Nb_2O_9$ b. $CaBi_2O_2(CO_3)_2$ and c. $Bi_4Ti_3O_{12}$. The vertical lines indicate the lines $0\,0\,z$ and $\frac{1}{2}\,\frac{1}{2}\,z$ in the unit cells. A denotes perowskitic ($E2_1$) regions in the structures.

It seemed of interest to investigate whether compounds could be synthesized with $m = 4$. The present investigation shows that structures of the above type with $m = 4$ exist.

Mixtures of Bi_2O_3, $BaCO_3$ and TiO_2, corresponding to the composition $BaBi_4Ti_4O_{15}$ were prepared and heated to 1100° C. Single crystals, thin plates, were picked out and Weissenberg photographs were taken. These could be interpreted by means of a body-centered tetragonal cell with $a = 3.86$ Å and $c = 41.7$ Å. The strong lines of the powder photographs (taken from preparations heated to 900° C (Au crucible) or 1100° C (Pt crucible) were easily identified since they could all be described with the aid of the "sub-lattice" ($a = 3.86$ $c = 41.7/9$). If the c axis were 9 fold even the weak lines could be explained (Table 3). In this way the cell edges were found to be $a = 3.864$ Å and $c = 41.76$ Å. The observed density was 7.2, which agrees fairly well with the assumption of 2 formula units/unit cell (calculated density 7.49).

The Weissenberg photographs registered $0kl$, $1kl$, hhl and h, $h+1$, l. In the Weissenberg and powder photographs there was nothing to indicate a lower Laue symmetry than $D_{4h}-4/mmm$. Except for the extinctions due to the body-centering, hkl occurring only for $h+k+l = 2n$, no systematic extinctions were found, which is characteristic of the space groups C_{4v}^{9}, $D_{2d}^{9,11}$ and D_{4h}^{17}. Fig. 2

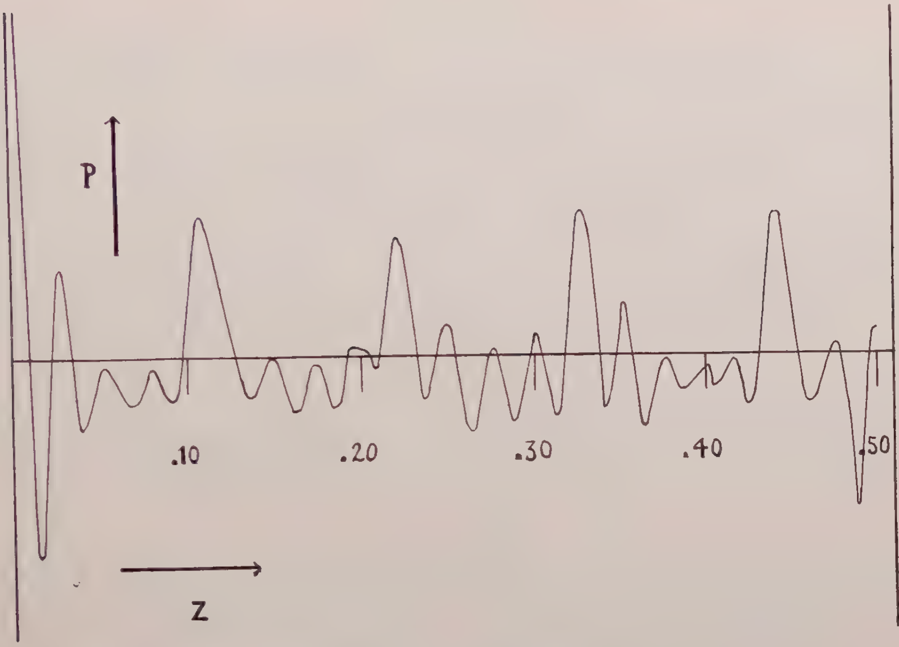

Fig. 2. Patterson Harker function of $BaBi_4Ti_4O_{15}$ along $0\,0\,z$. All observed reflexions were used for this calculation.

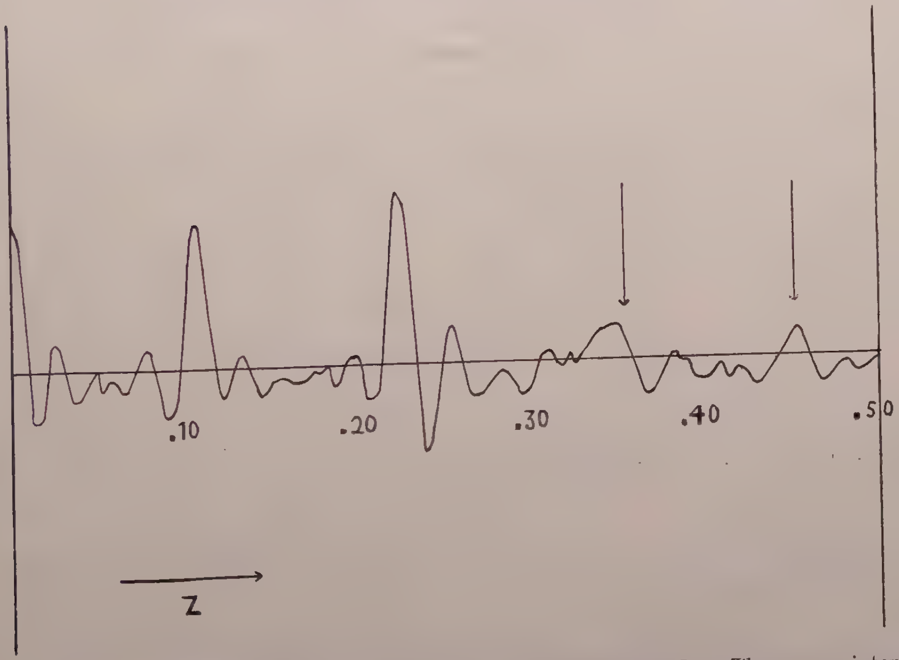

Fig. 3. Three dimensional Fourier cut along $0\,0\,z$ for $BaBi_4Ti_4O_{15}$. The same intensity material was used as for the Patterson Harker analysis pictured in Fig. 2. The signs of the amplitudes are the same as those obtained in the structure factor calculation for Table 2. The vertical arrows correspond to the z_{Ti} values actually assumed.

shows the Patterson Harker function along $0\,0\,z$. For all observed reflexions the F^2 values were estimated from: $F^2 \approx I_{\text{obs.}} \dfrac{\sin 2\,\theta}{1 + \cos^2 2\,\theta}$. As expected large peaks appear at $z \approx 1/9\ 2/9\ 3/9$ and $4/9$.

It is interesting to compare the cell dimensions found above with the ones which might be expected if the compound $BaBi_4Ti_4O_{15}$ is assumed to have a structure similar to that of $CaBi_2Nb_2O_9$ but with $m = 4$. In this case the a axis would have about the same value as found. The length of the c axis might be estimated in the following way: The c axis of Bi_3NbTiO_9 ($m = 2$ see (1)) is 25.11 Å; the value for $Bi_4Ti_3O_{12}$ ($m = 3$ see (2)) is 32.83, the difference is 7.72. If twice this value is added to the c axis of $BaBi_2Nb_2O_9$ ($m = 2$) the value 41.0 Å is obtained. The value actually found was, as mentioned above, 41.76 Å.

From the composition, cell dimensions, and crystal symmetry it seemed *a priori* probable that the structure of $BaBi_4Ti_4O_{15}$ was the one we anticipated. Therefore, the parameters were worked out with the aid of the parameters found for

Table 1

Weissenberg photographs of $BaBi_4Ti_4O_{15}$. Cu K_α radiation. For zero order photographs the regions of maximum absorption (see Wells (6)) are indicated by dotted lines. The intensities of 1 0 1, 1 0 3 and 1 0 5 have been taken from a zero order photograph, those of 1 0 27—1 0 49 from a first order photograph rotated around (1 0 0).

l	0 0 1	2 0 1	2 2 1	4 0 1	1 1 1	3 1 1	3 3 1
2		—	vw	—	—	—	vw
4		—	—	—	vw	—	vvw
6	vw	vvw	vw	—	w	—	vvw
8	m	m	w	—	m	vvw	vvw
10	m	m	w	—	st	vw	w
12	w	vvw	vw	—	vvw	—	w
14	w	—	vvw	—	w	—	—
16	st	w	m^+	vvw	m	vvw	m
18	vst	m	st	w	vst	w	st
20	st	w	w	vvw	w	—	—
22	m^+	vvw	vvw	vvw	vw	—	vw
24	w	vvw	vvw	vvw	vw	—	w
26	w	vvw	—	—	w	vvw	w
28	st	m^+	w^+	m	m	vw	
30	w	w	w	m^+	—	vw	
32	w	w^+	w		m	w	
34	m	m	m^+		m	m	
36	st	st	st		m^+	m	
38	vvw	—	—		w	—	
40	st	st	st		st	st	
42	vvw	—			vw		
44	—	—			—		
46	m	m			st		
48	w	m			st		
50	w^+				m		
52	w^+						

Table 1 (cont.)

Weissenberg photographs of $BaBi_4Ti_4O_{15}$. Cu K_α radiation.

l	101	211	301	321	411
1	m	w	vw	vw	—
3	vvw	vvw	—	vvw	—
5	vvw	—	—	—	—
7	w	vw	vvw	w	—
9	vst	st	m	m	w
11	—	—	—	—	—
13	w	—	—	—	—
15	w$^+$	—	vvw	—	—
17	m	vvw	vvw	—	—
19	st	vw	vvw	vvw	vvw
21	w	—	vvw	vvw	vvw
23	w	—	vvw	vvw	m
25	m$^+$	vw	vw	vw	m$^+$
27	st	m	w	m	
29	m	vw	vvw	vvw	
31	m	w	vw	vw	
33	vw	vvw	vvw	vw	
35	—	—	—		
37	m$^+$	m	m		
39	vw	w	m		
41	w	w	w$^+$		
43	w$^+$	m			
45	m	m$^+$			
47	—				
49	st				
51	—				

Bi_3NbTiO_9 and $Bi_4Ti_3O_{12}$. The positions of the heavy atoms might be expected to be: 2 Ba in $0\,0\,0$, 4 Bi in $\pm\,0\,0\,z_1$, 4 Bi in $\pm\,0\,0\,z_2$.

The positions of the Ti and O atoms might be expected to be:

4 Ti in $\pm\,0\,0\,z_3$, 4 Ti in $\pm\,0\,0\,z_4$, 2 O in $0\,0\,\frac{1}{2}$, 8 O in $\pm\,(0\,\frac{1}{2}\,(\frac{1}{2}-z_3)$, $\frac{1}{2}\,0\,(\frac{1}{2}-z_3))$, 8 O in $\pm\,(0\,\frac{1}{2}\,(\frac{1}{2}-z_4)$, $\frac{1}{2}\,0\,(\frac{1}{2}-z_4))$, 4 O in $\pm\,0\,0\,(z_3+z_4)/2$ 4 O in $\pm\,0\,0\,(z_3-(z_4-z_3)/2)$, 4 O in $0\,\frac{1}{2}\,\frac{1}{4}$, $\frac{1}{2}\,0\,\frac{1}{4}$

$z_1 \approx 1/9 \quad z_2 \approx 2/9 \quad z_3 \approx 0.350 \quad z_4 \approx 0.450$

These atomic positions would give rise to high peaks in the Patterson-Harker plot at the same values as actually found. The calculated area ratios agree, however, with the observed ones only in as much as the biggest area is found for the peak at 1/9. The reasons might be an incorrect choice of the zero level and errors in the estimation of the intensities.

The parameters were varied around the above values for different positions of the Ba atoms: 2 Ba in $0\,0\,0$, $0\,0\,z_1$ or $0\,0\,z_2$, 2 Ba equally distributed over $(0\,0\,0 + 0\,0\,z_1)$, $(0\,0\,0 + 0\,0\,z_2)$, $(0\,0\,z_1 + 0\,0\,z_2)$ or $(0\,0\,0 + 0\,0\,z_1 + 0\,0\,z_2)$. The best agreement seemed to be for $z_1 = 0.106 \pm 0.001$, $z_2 = 0.221 \pm 0.001$, $z_3 = 0.352 \pm 0.004$, $z_4 = 0.452 \pm 0.004$ with 2 Ba equally distributed over $(0\,0\,0 + 0\,0\,z_1 +$

Table 2

Weissenberg photographs of $BaBi_4Ti_4O_{15}$. Cu K_α radiation

Icalc. = $(2.5\ F/f_{Bi})^2$

l	Iobs. 1 0 1	Icalc. 1 0 1	Iobs. 2 1 1	Icalc. 2 1 1	Iobs. 3 0 1	Icalc. 3 0 1
1	m	73	w	90	vw	94
3	vvw	5.8	vvw	7.3	—	8.4
5	vvw	4.8	—	5.8	—	5.8
7	w	30	vw	31	vvw	32
9	vst	620	st	610	m	630
11	—	13	—	12	—	14
13	w	25	—	24	—	24
15	w^{+}	62	—	60	vvw	60
17	m	94	vvw	92	vvw	90
19	st	160	vw	170	vvw	170
21	w	15	—	12	vvw	12
23	w	19	—	18	vvw	18
25	m^{+}	120	vw	120	vw	120
27	st	320	m	320	w	320
29	m	45	vw	45	vvw	44
31	m	160	w	160	vw	160
33	vw	56	vvw	56	vvw	56
35	—	13	—	12	—	12
37	m^{+}	300	m	300	m	300
39	vw	12	w	13	m	13
41	w	15	w	14	w^{+}	15
43	w^{+}	200	m	200		
45	m	180	m^{+}	180		
47	—	18				
49	st	250				
51	—	3.6				

l	Iobs. 0 0 1	Icalc. 0 0 1	Iobs. 1 1 1	Icalc. 1 1 1	Iobs. 2 2 1	Icalc. 2 2 1
2			—	0.5	vw	0.6
4			vw	4.8	—	5.3
6	vw	21	w	23	vw	24
8	m	110	m	100	w	110
10	m	62	st	280	w	98
12	w	12	vvw	6.3	vw	9.6
14	w	14	w	16	vvw	14
16	st	83	m	79	m^{+}	81
18	vst	440	vst	480	st	450
20	st	170	w	36	w	120
22	m^{+}	100	vw	79	vvw	92
24	w	81	vw	86	vvw	78
26	w	39	w	36	—	39
28	st	280	m	240	w	280
30	w	2.6	—	11	w	1.4
32	w	11	m	20	w	12
34	m	140	m	110	m^{+}	140
36	st	240	m^{+}	240	st	240
38	vvw	19	w	35	—	21
40	st	380	st	230	st	340
42	vvw	11	vw	22		
44	—	3.2	—	4.8		
46	m	240	st	230		
48	w	69	st	46		
50	w^{+}	53	m	110		
52	w^{+}	280				

Table 3

Powder photographs of $BaBi_4Ti_4O_{15}$ Cr K_α radiation.

h k l	10^4 $\sin^2\theta$calc.	10^4 $\sin^2\theta$obs.	*I*obs.
0 0 10	753	745	w
1 0 1	887	878	m
1 0 5	1067	1058	vw
1 0 9	1489	1481	st
1 1 0	1758	1751	m
0 0 16	1927	1915	vvw
1 1 6	2031	2018	vw
1 1 8	2240	2236	vw
0 0 18	2438	2423	m$^-$
1 1 10	2511	2504	m$^+$
1 0 15	2572	2567	vvw
0 0 20	3010	2992	w
1 0 17	3054	3051	w
2 0 0	3516	3514	m
1 0 19	3596	3600	w$^+$
1 1 16	3685	3681	w
2 0 8	3998	3996	vw
1 1 18	4196	4186	m
1 0 21	4198		
2 0 10	4269	4265	w
2 1 1	4403	4402	vw
2 1 3	4418		
2 1 7	4764	4774	vw
1 1 20	4768		
2 0 14	4991	5009	m$^+$
2 1 9	5005		
2 0 16	5443	5440	vw
1 0 25	5582	5581	w
2 1 13	5667	5669	vvw
0 0 28	5900	5892	vw
2 0 18	5954	5960	m
2 1 15	6088	6089	vw
1 1 24	6093		
1 0 27	6365	6364	m
2 0 20	6526	6529	vw broad
2 2 0	7032	7070	w broad
2 2 2	7064		
2 1 19	7112		
3 0 5	8099	8104	vvw
1 0 31	8111		
2 2 14	8114		
2 1 23	8376	8365	vw
2 2 14	8507	8520	m
3 0 9	8521		
1 1 30	8531		
3 1 0	8802	8802	m
3 0 11	8812		
3 1 2	8820		
1 0 33	9074	9092	w broad
2 1 25	9098		
1 1 32	9464	9484	w broad
2 2 18	9470		

$0 0 z_2$). Other distributions of Ba should, however, not be excluded; as will be seen the three dimensional Fourier cut along $0 0 z$ (see Fig. 3) seems to favour the following Me arrangement: 4 Bi $\pm 0 0 z_2$ and (2 Ba + 4 Bi) equally distributed over the positions $0 0 0$ and $\pm 0 0 z_1$.

From Table 1 it is seen that roughly for the same value of l: $I_{00l} \approx I_{20l} \approx I_{22l} \approx I_{40l}$, $I_{11l} \approx I_{31l} \approx I_{33l}$, $I_{10l} \approx I_{21l} \approx I_{30l} \approx I_{32l} \approx I_{41l}$ as might be expected from the above atomic positions with atoms only on the lines $0 0 z$, $\frac{1}{2} \frac{1}{2} z$, $0 \frac{1}{2} z$ and $\frac{1}{2} 0 z$. Table 2 gives calculated and observed intensities for the rows $0 0 l$, $1 0 l$, $1 1 l$, $2 1 l$, $2 2 l$ and $3 0 l$. It was found from the Weissenberg photographs that $0 0 28 \gg 0 0 30$ and $1 0 37 > 1 0 39$ (see Table 2) but the observed ratios do not seem to be as large as those calculated. This is more clearly seen for the rows $4 0 l$ and $3 0 l$ where $4 0 28 < 4 0 30$ and $3 0 37 \geq 3 0 39$. These discrepancies could neither be removed by small variations in the z parameters nor by assuming other distributions of the Ba atoms for the structure factor calculations. Other discrepancies found from Tables 1 and 2 are $2 2 2 : 2 2 4$, $3 3 2 : 3 3 4$, $0 0 50 : 0 0 52$ and $1 1 48 : 1 1 52$. However, for most reflexions the agreement is quite good and considering the errors which might be introduced by absorption effects and errors in the ratios f_{Ti}/f_{Bi} and f_0/f_{Bi} the agreement might on the whole be classified as fairly good.

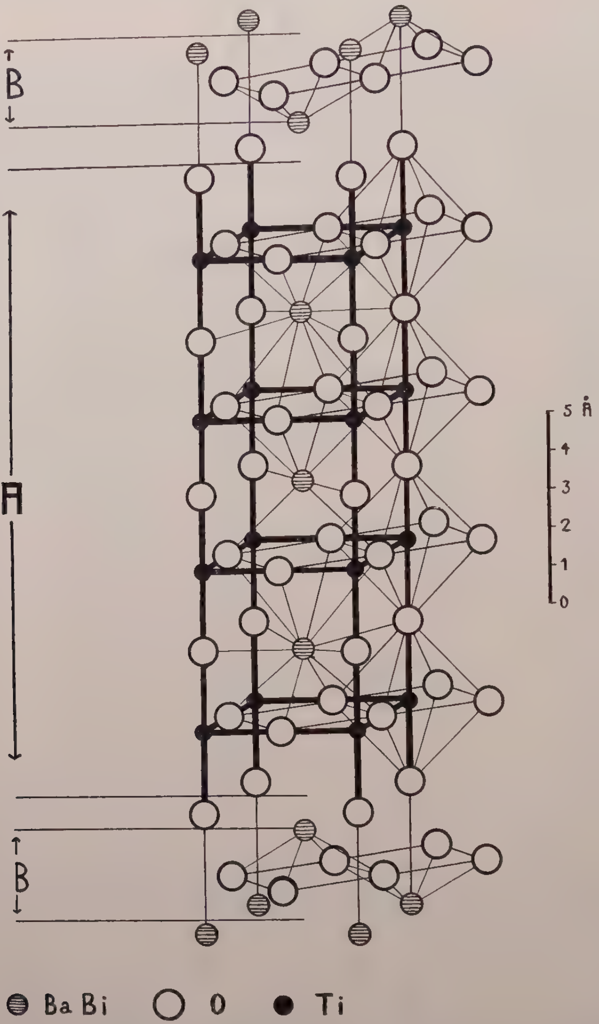

Fig. 4. One half of the unit cell of $BaBi_4Ti_4O_{15}$. A denotes the perowskitic region and B the Me_2O_2 layers.

The following structure is therefore proposed:
$D_{4h}^{17} - I4/mmm$
$(000,\ \frac{1}{2}\frac{1}{2}\frac{1}{2}) +$

$2\ Me_1$ in 2 (*a*) 000
$2\ O_1$ in 2 (*b*) $00\frac{1}{2}$
$8\ O_2$ in 8 (*g*) $0\frac{1}{2}z;\ 0\frac{1}{2}\bar{z};\ \frac{1}{2}0z;\ \frac{1}{2}0\bar{z}$ $z = 0.048$
$4\ Ti_1$ in 4 (*e*) $00z;\ 00\bar{z}$ $z = 0.452$
$4\ O_3$ in 4 (*e*) $z = 0.402$

4 Me_2 in 4 (*e*)	$z = 0.106$
8 O_4 in 8 (*g*)	$z = 0.148$
4 Ti_2 in 4 (*e*)	$z = 0.352$
4 O_5 in 4 (*e*)	$z = 0.302$
4 Me_3 in 4 (*e*)	$z = 0.221$
4 O_6 in 4 (*d*) $0 \frac{1}{2} \frac{1}{4}$; $\frac{1}{2} 0 \frac{1}{4}$	

Ba and Bi equally distributed over all Me positions.

If the structure is described by means of an orthorhombic space group (D_{2h}^{23}), as used for the structures of Bi_3NbTiO_9 and $Bi_4Ti_3O_{12}$, the positions will be: D_{2h}^{23} — F *m m m* (0 0 0; $\frac{1}{2} \frac{1}{2} 0$; $0 \frac{1}{2} \frac{1}{2}$; $\frac{1}{2} 0 \frac{1}{2}$) + 4 Me_1 in 4 (*a*) 0 0 0, 4 O_1 in 4 (*b*) $0 0 \frac{1}{2}$, 16 O_2 in 16 (*j*) $\frac{1}{4} \frac{1}{4} z$; $\frac{1}{4} \frac{1}{4} \bar{z}$; $\frac{1}{4} \frac{3}{4} z$; $\frac{1}{4} \frac{3}{4} \bar{z}$; $z = 0.048$, 8 Ti_1 in 8 (*i*) $0 0 z$; $0 0 \bar{z}$ $z = 0.452$, 8 O_3 in 8 (*i*) $z = 0.402$, 8 Me_2 in 8 (*i*) $z = 0.106$, 16 O_4 in 16 (*j*) $z = 0.148$, 8 Ti_2 in 8 (*i*) $z = 0.352$, 8 O_5 in 8 (*i*) $z = 0.302$, 8 Me_2 in 8 (*i*) $z = 0.221$, 8 O_6 in 8 (*f*) $\frac{1}{4} \frac{1}{4} \frac{1}{4}$; $\frac{1}{4} \frac{1}{4} \frac{3}{4}$.

The distances (Å) and coordination will be:

$O_1 - 8\ O_2 = 2.78$	$O_2 - 4\ O_2 = 2.73$	$Me_2 - 4\ O_2 = 3.10$	$Ti_1 - O_3 = 2.09$
$O_3 - 4\ O_2 = 2.84$	$O_4 - 4\ O_4 = 2.73$	$Me_2 - 4\ O_3 = 2.75$	$Ti_1 - 4\ O_2 = 1.93$
$O_3 - 4\ O_4 = 2.84$	$O_6 - 4\ O_6 = 2.73$	$Me_2 - 4\ O_4 = 2.61$	$Ti_1 - O_1 = 2.00$
$O_5 - 4\ O_6 = 2.91$			
	$Me_1 - 8\ O_2 = 2.78$	$Me_3 - 4\ O_6 = 2.28$	$Ti_2 - O_3 = 2.09$
	$Me_1 - 4\ O_1 = 2.73$	$Me_3 - 4\ O_5 = 2.89$	$Ti_2 - 4\ O_4 = 1.93$
		$(Me_3 - 4\ O_4 = 3.64)$	$Ti_2 - O_5 = 2.09$

One half of the unit cell is shown in Fig. 4.

I wish to thank Professor L. G. Sillén for valuable discussions on this work.

Stockholms Högskola, Institute of Inorganic and Physical Chemistry. May 1950.

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ARKIV FÖR KEMI Band 2 nr 38

Vorgelegt am 7. Juni 1950

Einflüsse von Wirkstoffen auf Lebensvorgänge normaler und sarkomtragender Ratten

Von Beth von Euler und Hans von Euler

Mit 1 Figur in Text.

Unter den Wirkstoffen, welche an normalen Ratten wichtige Lebensvorgänge beeinflussen, sind in erster Linie die Hormone zu nennen. Inwiefern sich in dieser Hinsicht tumortragende Tiere von normalen Tieren unterscheiden ist eine Frage, welche in dieser Allgemeinheit kaum beantwortet werden kann. Zieht man zunächst die Wachstumshormone in Betracht, so muss hervorgehoben werden, dass durch Einführung chemischer, auch mitosenhemmender Stoffe das Wachstum des Tumors und das Wachstum des ganzen Organismus gleichzeitig beeinflusst wird. Ein analoger Umstand erschwert auch oft die Beurteilung des Einflusses von wachstumbefördernden Vitaminen.

Sexualhormone (z. B. Östron), die in gewissen Geweben das Wachstum stimulieren, sollen auch in entarteten Geweben das Wachstum steigern, wenn sie auch im allgemeinen *nicht* imstande sind, maligne Degeneration der Zellen hervorzurufen, also, wie cancerogene Kohlenwasserstoffe, Krebs zu *erzeugen*.[1]

Der Einfluss von Sexualhormonen ist, wie auch der Einfluss der meisten Noxen, von der Grösse der Dose abhängig, und zwar aus verschiedenen Gründen, in der Regel wohl deshalb, weil die Reizwirkung kleiner Dosen auf das Abwehrsystem übertroffen wird von den Stoffwechsel-hemmenden Wirkungen grosser Dosen oder von den langdauernden oder ständigen Einflüssen von Ergonen, wie sie zustande kommen, wenn der canceröse Organismus selbst sie ausbildet.

Der Einfluss der Sexualhormone auf die Entwicklung der Tumoren kommt auch zum Ausdruck in der Beziehung, welche zwischen Krebswachstum und Gravidität besteht. Wir haben schon vor längerer Zeit mit Inez Rönnestam-Säberg[2] an einer Reihe gravider Ratten Impfungen mit Jensen-Sarkom vorgenommen, und fanden — mit 1 Ausnahme — die Entwicklungshemmung durch die Gravidität sehr erheblich. Das *Angehen* des Tumors wird *nicht* beeinflusst.

Unsere Ergebnisse stehen in Übereinstimmung mit den älteren Resultaten von Uhlenhuth[3], ferner von Graff, Fichera Baroni, sowie den neueren von Frank und Smith[4], welche angeben, dass das Tumorwachstum nach beendeter Schwangerschaft wieder zunimmt, und von Baatz.

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[1] Vgl. z. B. H. Friedrich-Freksa, Geburtshilfe u. Frauenheilkunde 3, 199 (1941).

[2] H. v. Euler, I. Rönnestam-Säberg u. B. v. Euler, Z. physiol. Chem. 270, 125 (1941). Siehe auch C. v. Euler u. H. v. Euler, Dieses Arkiv 14 B No 36 (1941).

[3] P. Uhlenhuth, Arb. a. d. Reichsges. Amt 30, 434 (1909).

[4] Frank u. Smith, Am. J. Obstetr. 34 616 (1937).

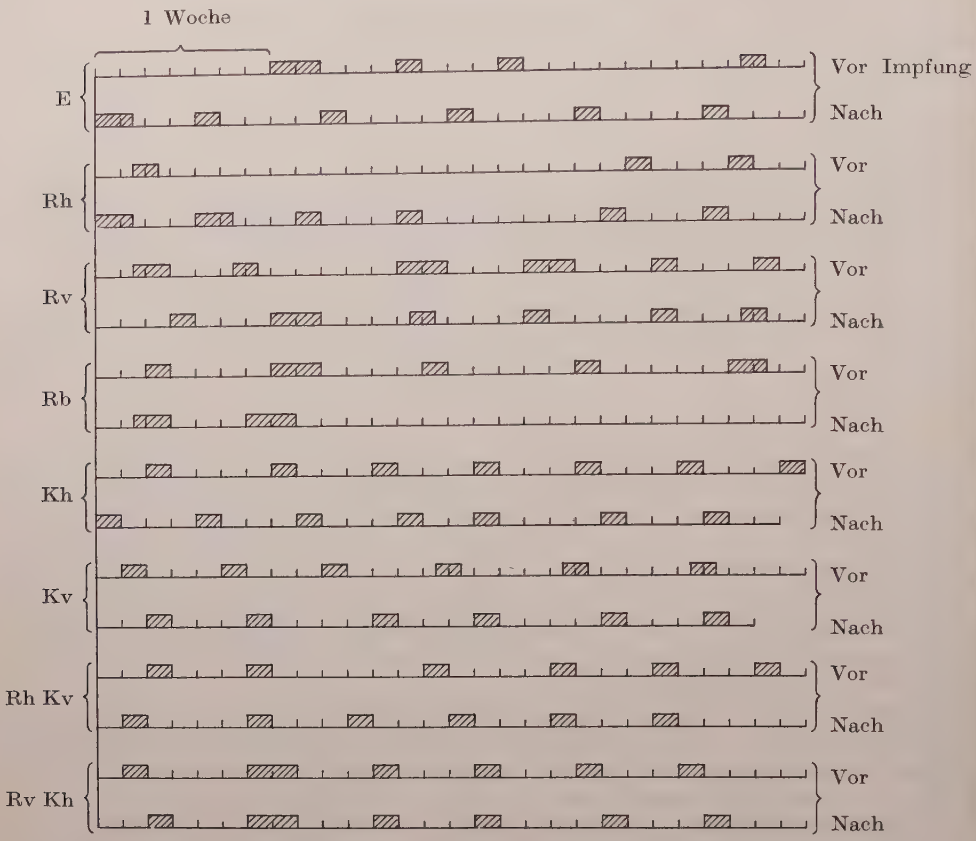

Fig. 1.

Wir haben in Betracht gezogen, „dass der unzweifelhaft bestehende tumorhemmende Einfluss der fortgeschrittenen Schwangerschaft mit der Ausschüttung von Hormonen in Zusammenhang zu setzen ist“.

Wenn es also feststeht, dass bei der Gravidität einerseits eine Ausschüttung von Follikulin, sowie eine Hemmung des Tumorwachstums eintritt, und andererseits Follikulin den Sexualcyklus bedingt[1], so liegt es nahe, einen Einfluss des cancerösen Zustandes auf den Sexualcyklus zu suchen.

Wir haben deswegen an einer Reihe von Ratten den Sexualcyklus durch die übliche Abstrichmethode *vor und nach der Impfung mit Benzpyrentumoren* während 28 Tagen verfolgt, und zwar mit Beginn 16 Tage nach der Impfung; die Tumoren hatten sich dann zu entwickeln begonnen.

Die Abstriche wurden einmal täglich entnommen, luftgetrocknet, mit Methylalkohol fixiert und dann mit Hämalaun-Eosin gefärbt.

Das Ergebnis wurde in der Weise festgelegt, dass der Eintritt und die Dauer des Östrus in einer Figur graphisch angegeben wurde. Die schraffierten Abschnitte jeder Zeile bedeuten also den Östrus-Zustand. (In der Figur sind 8 der untersuchten

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[1] Siehe Long u. Evans, Mem. Univ. of California, Vol. 6 (1922).

Ratten mit den Bezeichnungen unseres Laboratorium-Tagebuchs (E, Rh u. s. w.) versehen.)

Auch bei 3 weiteren untersuchten Tieren mit über 20 ccm grossen Tumoren war der Abstand zwischen den Östrus-Perioden gegenüber den normalen Tieren praktisch unverändert.

Aus der Figur ist ersichtlich, dass der Sexualcyklus der untersuchten Ratten durch die eingeimpften, wachsenden Benzpyrentumoren *keine* Änderung erfahren hat. Das den Sexualcyclus bestimmende Hormon wird also durch den subcutan wachsenden Benzpyrentumor nicht beeinflusst.

Übertragbarkeit der Tumor-Resistenz auf die Nachkommen

Zur Untersuchung, ob die Nachkommen von Ratten, welche Benzpyren-Impftumoren spontan zurückgebildet haben, erblich gegen den gleichen Tumor resistent sind, wurden 7 solche Ratten, nämlich 5 Weibchen und 2 Männchen, gepaart.

Das Resultat war folgendes:

Von 18 mit Benzpyrentumoren geimpften Nachkommen bildeten sich
in 15 Tieren „Stetig wachsende Tumoren“ aus,
bei 3 Tieren waren die Implantate nicht angegangen,
bei keinem der Tiere waren die Tumoren retrograd.

Die Entwicklung der Tumoren der Eltern ist durch folgende Zahlen gekennzeichnet:

		Max. Grösse	Spätere Beobachtungen	
Männchen:	5 Wochen	2,2 ccm	$2^1/_2$ Monate	kleine Spur
	3 Wochen	0,4 ccm	1 Monat	—
Weibchen:	4 Wochen	1,0 ccm	5 Wochen	„grosse Spur“
	4 Wochen	kleine Bohne	5 Wochen	Spur
	3 Wochen	1,3 ccm	5 Wochen	Spur
	6 Wochen	6,0 ccm	8 Wochen	deutl. retrograd
	3 Wochen	Haselnuss	6 Wochen	Spur

Es ergibt sich also das recht überraschende Resultat, dass die Resistenz gegen Benzpyrentumoren keineswegs als erbliche Eigenschaft hervortritt. Offenbar hat die Virulenz des eingeimpften Tumors einen ganz überwiegenden Einfluss auf das Angehen und die Entwicklung des Implantates.

Untersuchung der Resistenz von Ratten mit normalen Jensensarkomen gegen Injektion von Benzpyren-Öl

Die zu diesen Versuchen verwendeten Jensensarkome erwiesen sich zu dieser Zeit so wenig virulent, dass alle überhaupt angegangenen Impfsarkome retrograd wurden.

Die Injektion jeder der 3 Versuchstiere erfolgte während das retrograde Jensensarkom noch etwa Bohnen-gross war. Etwa 4 Monate nach der Injektion des Benzpyrens begann die Entwicklung des Benzpyren-Tumors bei allen 3 Tieren; die Tumoren wuchsen, wie die nachfolgenden Zahlen zeigen, mit verschiedener Geschwindigkeit.

Grösse des Sarkoms nach dem fünften Monat.

Ratte I	Ratte II	Ratte III
grosse Haselnuss	kleine Bohne	11 g

Es ergibt sich also, dass *die vorhergehende Überwindung eines Jensensarkoms die Entwicklung eines Tumors durch Benzpyren-Emulsion nicht hindert.*

Injektionen von oxydierenden und reduzierenden Stoffen in sarkomtragende Ratten

Seit längerer Zeit werden in diesem Institut die Einflüsse injizierter Stoffe studiert. Unter den untersuchten Stoffklassen sind zu erwähnen:

1) bakteriostatische Stoffe, bearbeitet in Zusammenhang mit Studien über Streptomycin[1] und über Sulfanilsäure-Reduktonkomplexe.
2) Mitose-hemmende Stoffe, Colchicin und Trypaflavin.[2]
3) Sarkomextrakte, um in voll entwickelten Tumorgeweben vorkommende Hemmungskörper auf die in der Entwicklung befindlichen Gebilde canceröser Zellen zu übertragen.[3]

Die Mehrzahl unserer noch nicht publizierten Untersuchungen schliesst sich an die Feststellung[4] an, dass Tumoren ausserordentlich arm an Cytochrom, an Cytochrom-Oxydase und an Diaphorase sind. Diese, wie wir glauben, wesentliche Tatsache hat uns verschiedentlich zu Versuchen angeregt, den oxydo-reduktiven Stoffwechsel durch Einführung chemischer Oxydationsmittel und Reduktionsmittel in den lebenden Organismus zu beeinflussen.[5] Unser Ziel liegt in der Beeinflussung der Acidität und des Oxydo-Reduktionspotentiales, und zwar

a) im gesammten Organismus, um dadurch eine krebsige Mutation der normalen Zellen zu hemmen,
b) im wachsenden Krebsgewebe, um den Stoffwechsel desselben zu hemmen und die Entwicklung des normalen Gewebes zu fördern.

Dehydrogenasen in Sarkomgeweben nach Injektion oxydierender und reduzierender Stoffe

In dialysierten Extrakten von Sarkomgeweben (Jensensarkome: Mai–September 1948) wurden mit der Thunberg-Methodik starke Wirkungen von Robisonester-Dehydrogenase (A) und von Succino-Dehydrogenase (B) nachgewiesen, siehe Tabelle S. 533.

Wir haben diese Sarkomratten teils intramuskular teils intratumoral injiziert und zwar während 3 Wochen 3mal wöchentlich; die injizierten Volumina betrugen intramuskular 0,2 ml, intratumoral 0,1 ml, und die Mengen der injizierten Stoffe betrugen, auf 9 Injektionen verteilt:

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[1] EULER, Dieses Arkiv 25 A No 8 (1947). und 26 A No 8 (1947).
[2] EULER u. W. SOLODKOWSKA, Dieses Arkiv 24 A No 24 (1947).
[3] EULER u. M. JAARMA, Dieses Arkiv 23 A No 17 (1946) und 25 A No 7 (1947).
[4] EULER u. HARRY HELLSTRÖM, Z. physiol. Chem. 260, 163 (1939).
[5] Siehe hierzu auch H. W. SCHMIDT, Z. Krebsforsch. 56, 143 (1948).

4,5 mg Benzochinon	6,8 mg $ZnCl_2$
6,2 mg Na-Tartrat	9,0 mg Ascorbinsäure
5,5 mg Na-Fe^{III} Tartrat	3,0 mg Hydrochinon

Totalvolumen im Thunberg-Rohr: 2,5 ccm. Gemischt wurden 0,50 ccm 0,10 mol. Phosphatpufferlös. + 0,50 ccm Organextrakt + 1,0 ccm Substratlös. (bei Verwendung von Robisonester) + 0,5 ccm Cozymase-Lös. = 30 γ Cozymase.

Dazu 0,50 ccm Mb-Lös. 1 : 5000. Temp. 30° pH = 7,5.

Vorbehandlung der Ratten mit	Entfärbungszeit in Min.	
	A	B
Ohne Vorbehandlung	79	54
Benzochinon	86	—
Na-Tartrat	72	60
Na-Fe-Tartrat	92	—
$ZnCl_2$	61	44
Ascorbinsäure	67	59
Hydrochinon	108	90

Gewebe-Acidität des Jensen-Sarkoms nach Injektion von oxydierenden und reduzierenden Stoffen in vitro

Ausgehend von der Annahme, dass das Oxydo-Reduktionspotential sowie die Acidität entscheidende Faktoren bei der Entwicklung des Tumorgewebes ausmachen, haben wir zunächst untersucht, welchen Einfluss Injektionen oxydierender und reduzierender Stoffen in das lebende Sarkomtier auf die *Acidität* des *Jensen-Sarkoms* ausüben.

Die Injektionen geschahen stets intramuskular und zwar in gleicher Weise und mit den gleichen Mengen wie bei den obigen Bestimmungen der Dehydrase-Wirkungen.

20–26 Stunden nach den Injektionen wurden die Tiere getötet und die Sarkome sofort operativ entfernt und von Nekrosen befreit. Im Verlauf der nächsten Stunde wurde die Aciditätsmessung am frischen Sarkom vorgenommen, und zwar an 2 Stellen durch direktes Einstechen der Elektroden in das Gewebe. (H. HASSELQUIST u. A. GLASER).

In der folgenden Tabelle sind die Ergebnisse einiger orientierender Versuche angegeben.

Vorbehandlung des Sarkomtieres mit	pH
Benzochinon	6,67
Ascorbinsäure	7,00
Cobaltchlorid	6,32
Zinkchlorid	6,82
Hydrochinon	6,07

Diese orientierenden Versuche liefern noch keinen Anhaltspunkt, in *welcher Weise* die Änderung der Acidität und der Reduktionspotentiale durch die Vorbehandlung zustande kommt, also welcher Stoff bezw. welche Stoffe das gemessene pH des Sarkoms bestimmen. In der Fortsetzung der Untersuchung soll auch versucht werden, die aufgenommenen Metallspuren analytisch zu ermitteln.

Stockholm, Universität, Vitamininstitut.

Tryckt den 14 oktober 1950

Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

ARKIV FÖR KEMI Band 2 nr 39

Communicated 24 May 1950 by Arne Westgren and Percy Quensel

The crystal structure of $Th(OH)_2SO_4$

By Georg Lundgren

With 6 figures in the text

In connexion with investigations of the hydrolysis of Th^{4+} (S. Hietanen, to be published), Lundgren and Sillén (1949) determined the crystal structure of $Th(OH)_2CrO_4H_2O$. They found that it is built up of infinite chains of composition $[Th(OH)_2]_n^{2n-}$, joined by CrO_4^{2-} groups. Each chain consists of a zigzag row of thorium atoms, linked together by OH^- ions forming two parallel straight rows (*cf.* Fig. 2). This structure element was not previously known. In other hydroxy or oxy salts there are two-dimensional sheets of metal ions linked together by oxygen atoms. For instance Bi^{3+} and La^{3+} form sheets of composition $(BiO)_n^{n-}$ and $(LaO)_n^{n+}$ (Bannister and Hey 1935; Sillén 1940, 1942; Lagercrantz and Sillén 1948; Sillén and Nylander 1941; Sillén and Lundborg 1943). Similar sheets seem to occur in solutions, too (Granér and Sillén 1947). Somewhat different examples of crystals containing such two-dimensional networks are the uranates of barium, strontium and calcium (Samson and Sillén 1948; Zachariasen 1948 a). In these crystals UO_2^{2+} ions are joined by oxygen bridges into infinite planes with average composition $(UO_4)_n^{2n-}$.

However, to see if the $[Th(OH)_2]_n^{2n+}$ chain in $Th(OH)_2CrO_4H_2O$ is an essential structure element, and thus also occurring in crystals of other thorium hydroxy compounds, it seemed desirable to investigate the crystal structure of some other salt $Th(OH)_2X_2$. A suitable substance for this purpose should contain as little water of crystallization as possible and preferably be of a rather high symmetry.

Gmelin-Kraut's Handbuch (1928) gives several salts of formula $Th(OH)_2X_2$, but unfortunately most of them contain a great deal of water of crystallization. However, a "basic" thorium sulphate of composition $ThOSO_4H_2O$ or $Th(OH)_2SO_4$ is also described and is said to be easy to obtain in a crystalline form (Hauser and Wirth 1908). This salt thus seemed rather promising from my point of view and was chosen as a suitable object for the investigation.

Preparation of the crystals

The crystals were prepared in principle as described by Hauser and Wirth (1908).

Thorium sulphate was prepared by adding concentrated sulphuric acid to a saturated solution of thorium nitrate. The precipitate formed was filtered off and washed with water. From the thorium sulphate obtained in this way a solution saturated at room temperature was prepared. When 50 ml of this solution was diluted with 50 ml of water and heated in an autoclave to 160—180 °C for about a week, good crystals for X-ray work were obtained. However, if an excess of sulphuric acid was added to the solution, thread-like crystals of a neutral thorium sulphate [probably $Th(SO_4)_2(H_2O)_4$] were obtained and yet another phase from solutions containing only a small excess of sulphuric acid. To avoid contamination with crystals of other thorium sulphates the solution was neutralized with dilute ammonia before the heating, until the precipitate formed immediately after each addition of ammonia barely dissolved on shaking.

After heating the autoclave was allowed to cool, and the crystals were filtered off and dried in air. Under the microscope they proved to be colourless, transparent plates about 0.1—0.2 mm in length. However, they had often grown together into long, saw-like bands or into white irregular clusters, as described by HAUSER and WIRTH (*l. c.*).

Analysis

After drying in an oven at 100 °C, the sample was dissolved in 4 C HNO_3, and the amount of *sulphate* determined gravimetrically by precipitation as $BaSO_4$. In the filtrate thorium hydroxide was precipitated with ammonia, and the amount of *thorium* determined after ignition and weighing as ThO_2.

The *water* was determined by HARTWIG-BENDIG's (1941) modification of BRUSH (1868) and PENFIELD's (1894) method, given by KOLTHOFF and SANDELL (1943).

The density of the crystals was determined from the loss of weight in benzene.

	Found		Calculated for $Th(OH)_2SO_4$
% SO_3	22.5	22.4	22.11
% ThO_2	72.3	72.3	72.92
% H_2O	5.9	5.7	4.97
Density	4.6_5	4.7_0	4.81 (for 4 formula units per cell)

Unit cell and space group

A crystal was set with a diagonal in the plane of the plate as rotation axis, and rotation and WEISSENBERG photographs ($h\,0\,l$ and $h\,1\,l$) were taken, using Cu Kα-radiation. From these and from a LAUE photograph of the same crystal it was concluded that the crystal has LAUE symmetry $m\,m\,m$ (orthorhombic), and that the dimensions of the unit cell are about 11.7_4, 6.0_3, and 7.0_9 kX (the longest axis being perpendicular to the plate). More accurate values of the dimensions of the unit cell were calculated from powder photographs, taken with Cr Kα-radiation in focussing cameras of PHRAGMÉN type (Table 1):

$$a = (11.709 \pm 0.005)\ \text{kX} = (11.733 \pm 0.005)\ \text{Å}$$
$$b = (6.028 \pm 0.005)\ \text{kX} = (6.040 \pm 0.005)\ \text{Å}$$
$$c = (7.045 \pm 0.005)\ \text{kX} = (7.059 \pm 0.005)\ \text{Å}$$
$$V = 497.2_5\ \text{kX}^3 = 500.2_7\ \text{Å}^3.$$

Table 1

Powder photographs of $Th(OH)_2SO_4$

$CrK\alpha_1$-radiation. $\lambda = 2.285$ kX $= 2.290$ Å.

h k l	$10^4 \sin^2 \theta$ calc	$10^4 \sin^2 \theta$ obs	*I* calc	*I* obs
2 1 1	1003	1001	31	w+
0 0 2	1052	—	1	—
3 0 1	1120	1118	5	vw
1 0 2	1147	1146	3	vvw
2 0 2	1433	1434	1	w
0 2 0	1436		25	
3 1 1	1479	(1480)	36	(m)
1 1 2	1506	1509	79	m
4 0 0	1523	—	4	—
4 0 1	1786	1790	21	m$^-$
2 1 2	1792		9	
1 2 1	1795		11	
2 2 0	1818	1824	22	vw
4 1 0	1883	1886	55	m
3 0 2	1909	1915	16	vw
2 2 1	2081	2083	32	w
4 1 1	2146	(2148)	1	(vvw)
3 1 2	2268	2276	6	vvw
1 0 3	2462	2456	42	w+
0 2 2	2489	(2490)	0	(vvw +)
3 2 1	2557	2556	7	vvw
4 0 2	2575	2586	1	w+
1 2 2	2584		32	
5 0 1	2643	2643	8	w
0 1 3	2726	2730	7	vvw
2 0 3	2748	—	1	—
1 1 3	2821	2816	12	vvw
2 2 2	2870	—	3	—
4 1 2	2935	2938	8	vvw
4 2 0	2960	—	2	—
5 1 1	3002	(3005)	25	(m)
2 1 3	3107	3107	5	vvw$^-$
4 2 1	3223	(3227)	83	(st)
3 0 3	3224		9	
3 2 2	3346	(3344)	90	(m +)
6 0 0	3428	3439	12	m+
5 0 2	3432		60	
0 3 1	3496	3500	41	m
3 1 3	3583	3582	64	m
1 3 1	3592		13	
2 3 0	3614	(3619)	21	(w +)
6 0 1	3691	3696	10	w
6 1 0	3787	3797	25	m +
5 1 2	3791		23	
2 3 1	3877	3893	27	m
4 0 3	3890		3	
1 2 3	3899		90	
4 2 2	4012	—	0	—
6 1 1	4050	(4049)	44	(m +)
5 2 1	4080	(4078)	4	(vvw)
2 2 3	4185	4191	18	vw
0 0 4	4208		23	
4 1 3	4249	(4241)	3	(vw)
1 0 4	4303	—	2	—
3 3 1	4353	4348	35	w
1 3 2	4380	4373	69	m
6 0 2	4479	4482	2	vvw$^-$
2 0 4	4589	4592	16	vvw
3 2 3	4661	4665	5	vw
1 1 4	4662		26	
2 3 2	4666		15	
5 0 3	4747	4756	11	w +
4 3 0	4757		48	
6 1 2	4840	—	3	—
6 2 0	4865	4875	23	m+
5 2 2	4869		87	
7 0 1	4928	4928	28	w+
2 1 4	4948		40	
4 3 1	5020	—	0	—
3 0 4	5065	(5044)	18	(vw)
5 1 3	5106	5118	74	w
6 2 1	5128		23	
3 3 2	5142		12	
7 1 1	5288	(5282)	4	(vvw)
4 2 3	5327	5333	12	vvw
3 1 4	5424	5430	9	vvw$^-$
0 3 3	5600	—	5	—
0 2 4	5645	5652	26	vvw
1 3 3	5695	—	6	—
7 0 2	5717	—	8	—
4 0 4	5731	—	0	—
1 2 4	5740	—	9	—
0 4 0	5748	(5757)	46	(w +)
6 0 3	5794	—	1	—
4 3 2	5809	—	6	—
5 3 1	5877	5886	28	vvw
6 2 2	5917	(5932)	12	(vvw)
2 3 3	5981	—	6	—
2 2 4	6026	6031	27	vvw
7 1 2	6076	6095	61	st
4 1 4	6090		74	
8 0 0	6093		17	
1 4 1	6106	(6112)	22	(st)
2 4 0	6129	(6129)	24	(vw)
6 1 3	6154	6158	11	vvw
5 2 3	6184	6192	13	vvw
8 0 1	6356	—	1	—
7 2 1	6365	6376	37	w
2 4 1	6392	(6398)	33	(w)

Table 1 (cont.)

$h\,k\,l$	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	I calc	I obs	$h\,k\,l$	$10^4 \sin^2\theta$ calc	$10^4 \sin^2\theta$ obs	I calc	I obs
8 1 0	6453	6461	2	w	4 2 4	7168	(7177)	1	(vvw)
3 3 3	6457		61		2 4 2	7181	(7177)	4	(vvw)
3 2 4	6501	(6478)	33	(vvw)	6 2 3	7231	—	1	—
5 0 4	6588	—	9	—	4 4 0	7272	(7273)	2	(vvw^{-})
6 3 0	6661	6669	21	w^{-}	2 1 5	7315	7319	41	vw
5 3 2	6666		22		7 1 3	7391	7393	8	vvw
1 0 5	6670		2		3 0 5	7431	(7430)	0	(vw)
8 1 1	6716	6725	67	m	8 1 2	7505	—	0	—
0 4 2	6800	—	1	—	8 2 0	7530	7537	45	m+
3 4 1	6868	6874	8	vvw	4 4 1	7535		55	
1 4 2	6895	6896	8	vvw	1 3 4	7536		18	
6 3 1	6924	6935	32	w	6 0 4	7635	7658	28	w
0 1 5	6934		55		3 4 2	7657		52	
5 1 4	6947	—	2	—	6 3 2	7711	—	1	—
2 0 5	6955	(6957)	21	(w^{-})	3 1 5	7791	—	0	—
1 1 5	7029	7032	0	w	8 2 1	7793	—	2	—
7 0 3	7032		42		2 3 4	7821	(7822)	42	(vw)
4 3 3	7124	(7119)	3	(vvw)	9 0 1	7975	7986	11	m^{-}
8 0 2	7145	7155	7	vw	5 3 3	7980		96	
7 2 2	7154		6		6 1 4	7995		27	

The photographs were measured and interpreted up to $\sin^2\theta = 0.97$.

The horizontal lines mark the boundaries between the angular ranges of the three different focussing cameras used.

Reflections systematically absent in space group D_{2h}^{16} have been omitted. Reflections coinciding with α_2 or β reflections are given in brackets.

The intensities were calculated from the expression $100 \cdot \frac{p}{8}\left(\frac{F}{4\,f_{\mathrm{Th}}}\right)^2$, where p is the number of equivalent reflections. In the photographs there is an orientation effect, favouring reflections with high h.

The mean value of the density is 4.6_8, so that there must be $3.8_9 \approx 4$ formula units $Th(OH)_2SO_4$ in the unit cell.

The reflections systematically absent are

$$h\,k\,0 \text{ with } h = \text{odd}$$

$$0\,k\,l \text{ with } k + l = \text{odd}.$$

This is characteristic of the two space groups D_{2h}^{16} and C_{2v}^{9}. Since it was possible to assign a plausible structure assuming D_{2h}^{16}, the space group of higher symmetry, the low-symmetrical C_{2v}^{9} was not considered.

In D_{2h}^{16} the following point positions are possible:

4 (a): $0\,0\,0;\ 0\,\tfrac{1}{2}\,0;\ \tfrac{1}{2}\,0\,\tfrac{1}{2};\ \tfrac{1}{2}\,\tfrac{1}{2}\,\tfrac{1}{2};$ 4 (b): $0\,0\,\tfrac{1}{2};\ 0\,\tfrac{1}{2}\,\tfrac{1}{2};\ \tfrac{1}{2}\,0\,0;\ \tfrac{1}{2}\,\tfrac{1}{2}\,0;$

4 (c): $\pm\,(x, \tfrac{1}{4}, z);\ \pm\,(\tfrac{1}{2} + x, \tfrac{1}{4}, \tfrac{1}{2} - z);$

8 (d): $\pm\,(x, y, z);\ \pm\,(\tfrac{1}{2} + x, \tfrac{1}{2} - y, \tfrac{1}{2} - z);\ \pm\,(\bar{x}, \tfrac{1}{2} + y, \bar{z});\ \pm\,(\tfrac{1}{2} - x, \bar{y}, \tfrac{1}{2} + z).$

Positions of the thorium atoms

To determine the positions of the thorium atoms the function

$$P(u, w) = \sum_h \sum_l I_{h0l} \cos 2\pi (hu + lw) \approx \sum_h \sum_l F^2_{h0l} \cos 2\pi (hu + lw) = P(u, p, w)$$

was calculated using the observed intensities of the WEISSENBERG photograph $h0l$. This function is approximately the projection on the uw plane of the PATTERSON function $P(u, v, w)$. In Fig. 1 it is drawn for $0 \leqq u \leqq \frac{1}{2}$, $0 \leqq w \leqq \frac{1}{2}$.

Since there are only four thorium atoms in the unit cell, they must occupy one of the fourfold positions. If they were situated in 4 (a) or 4 (b), $P(u, w)$ must have high peaks at $u = 0$, $w = 0$, and at $u = \frac{1}{2}$, $w = \frac{1}{2}$. $P(u, w)$ has, however, no maximum at $u = \frac{1}{2}$, $w = \frac{1}{2}$, and thus the thorium atoms are situated in 4 (c).

In the point position 4 (c) the interatomic distances are:

$$\text{I.}\quad \begin{aligned} u &= \pm 2x \\ w &= \pm 2z \end{aligned} \quad \text{(weight 1)}$$

$$\text{II.}\quad \begin{aligned} u &= \pm (\tfrac{1}{2} - 2x) \\ w &= \tfrac{1}{2} \end{aligned} \quad \text{(weight 2)}$$

$$\text{III.}\quad \begin{aligned} u &= \tfrac{1}{2} \\ w &= \pm (\tfrac{1}{2} - 2z) \end{aligned} \quad \text{(weight 2)}$$

Now, there are two very high peaks in $P(u, w)$ at $u = 0.37_5$, $w = \frac{1}{2}$ (A in Fig. 1) and at $u = \frac{1}{2}$, $w = 0.20_0$ (B in Fig. 1). They certainly correspond to the Th — Th vectors II and III above, and thus the x and z parameters for the thorium atoms are $x = 0.06_3$ and $z = 0.15_0$. These values demand a peak (I above) in $P(u, w)$ at $u = 0.12_6$, $w = 0.30_0$. The height of this peak should be about half that of the two others. As $P(u, w)$ has a peak of the right height at this point (C in Fig. 1), we conclude:

$$4\ \text{Th in } 4\,(c)\text{:}\ x = 0.06_3,\ z = 0.15_0.$$

The remaining maxima in $P(u, w)$ were not considered, as the peaks due to Th — O and Th — S vectors are of about the same height as the false maxima formed by errors in the calculation of $P(u, w)$, e. g. diffraction effects, or errors in the observed intensities due to absorption in the crystal (WELLS 1937).

Positions of the light atoms

General remarks

The positions of the oxygen and sulphur atoms could not be determined from the intensities but had to be found from considerations of the interatomic distances.

The sulphate group was considered to be a regular tetrahedron. Different values are reported in the literature for the bond lengths S—O and O—O. ZACHARIASEN and ZIEGLER (1932) give S—O = 1.4_9 Å and O—O = 2.4_3 Å for Na_2SO_4, and ZACHARIASEN (1932) found S—O = 1.4_4, 1.4_5, and 1.4_9 Å and

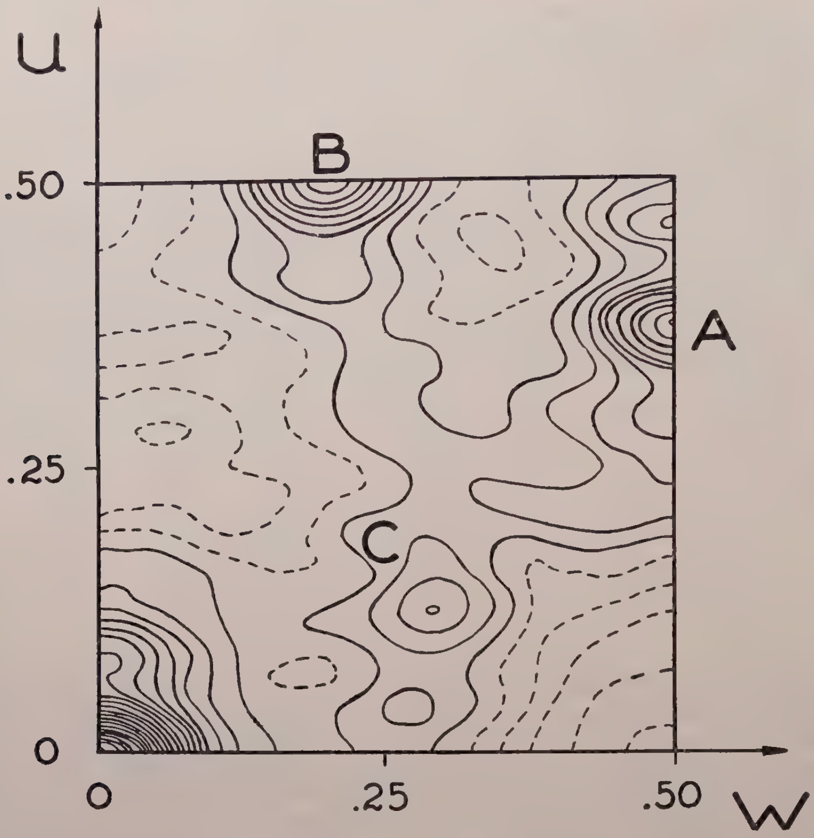

Fig. 1. PATTERSON analysis of $Th(OH)_2SO_4$.

The function $P(u, w) = \sum_h \sum_l I_{h0l} \cos 2\pi (hu + lw) \approx P(u, p, w)$.

Dotted lines indicate negative values.

O—O = 2.4_1, 2.4_1, and 2.4_9 Å in $K_2S_2O_5$. By means of FOURIER synthesis BROWN and COX (1940) determined the bond lengths in KSO_3NH_2 to S—O = = 1.44 ± 0.03 Å and O—O = 2.3_9—2.3_6 Å. WELLS (1949) thought that BROWN and COX's determination gives the best value for the distance S—O in a tetrahedral ion. Thus it seems reasonable to consider the sulphate group as a regular tetrahedron with the sulphur atom situated at its centre and with the distance O—O = 2.4_0 Å, and thus S—O = 1.4_7 Å.

The distance between two oxygen atoms, not belonging to the same sulphate group, was assumed to be not less than 2.6_0 Å, and the distance Th—O not less than 2.4_0 Å. (In ThO_2, which has the CaF_2 structure, GOLDSCHMIDT and THOMASSEN (1923) found a coordination Th—8 O and a bond length Th—O = = 2.41 Å. The size of its unit cell was redetermined accurately by ZACHARIASEN (1948 b), who found Th—O = 2.42 Å.)

With these assumptions the space free for the oxygen atoms will be reduced as follows:

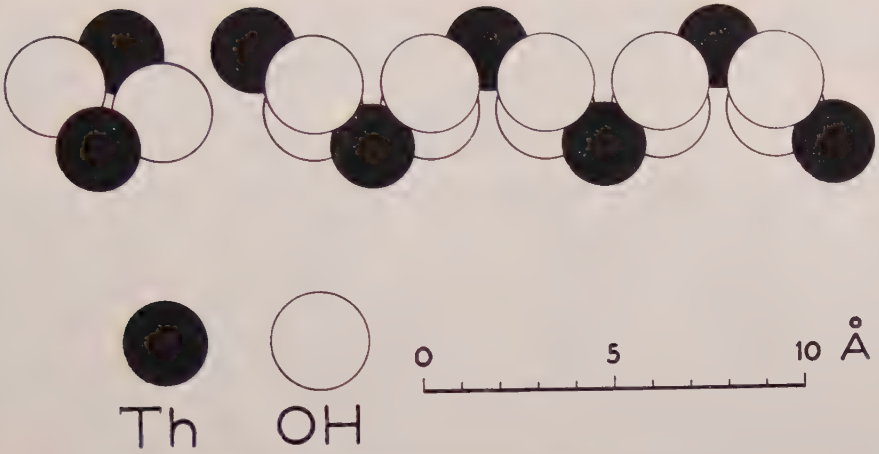

Fig. 2. The $[Th(OH)_2]_n^{2n+}$ chain in $Th(OH)_2CrO_4H_2O$ and $Th(OH)_2SO_4$ viewed along [0 1 0] (left) and from the side (right).

The centre of an oxygen atom can be situated either in a symmetry plane or at a distance $\geqq 1.3_0$ Å (1.2_0 Å for a sulphate oxygen atom) from it. As there are two symmetry planes at $y = \pm \frac{1}{4}$ in the unit cell the y coordinates for the oxygen atoms are within the rather narrow limits: $-0.04 \leqq y \leqq 0.04$, $y = 0.25$, $0.46 \leqq y \leqq 0.54$, and $y = 0.75$ (for the sulphate oxygen atoms: $-0.05 \leqq y \leqq 0.05$, $y = 0.25$, $0.45 \leqq y \leqq 0.55$, $y = 0.75$).

For the same reason a sphere with radius 1.3_0 (1.2_0) Å around each symmetry centre [the point positions 4 (a) and (b)], a sphere with radius 2.6_0 (2.4_0) Å around each oxygen atom that has been located, and a sphere with radius 2.4_0 Å around each thorium atom enclose domains forbidden for the centres of other oxygen atoms.

Positions of the non-sulphate oxygen atoms

From the function $P(u, w)$ (Fig. 1) it was concluded that the thorium atoms are situated in 4 (c) with $x = 0.06_3$ and $z = 0.15_0$. They thus form zigzag rows parallel to the y axis. The shortest distance between two thorium atoms in such a row is 3.9_8 Å, and its projection on the xz plane 2.5_8 Å. Now, Lundgren and Sillén (1949) have found a similar arrangement in $Th(OH)_2CrO_4H_2O$. This structure contains infinite chains of composition $[Th(OH)_2]_n^{2n+}$, formed by a zigzag row of thorium atoms held together by two almost parallel lines of OH^- ions (Fig. 2). The shortest distance between two thorium atoms in these chains is 4.0_2 Å, and its projection on a plane perpendicular to the direction of the chains 2.6_0 Å. As the difference between these two figures and the corresponding figures for $Th(OH)_2SO_4$ is very small, it seems reasonable to assume that in the latter crystal also there are such chains, running in the direction of the y axis. We thus conclude:

$$8\ O_{Th}\ (OH)\ \text{in}\ 8\ (d)\text{:}\ x = 0.40,\ y \approx 0,\ z = 0.39.$$

(The x and z coordinates were calculated under the assumptions that the oxygen atoms are situated exactly in the planes $y = 0$ and $y = \frac{1}{2}$, and that every oxygen atom is in direct contact with its two neighbouring thorium atoms.)

Positions of the sulphate groups

If the crystal belongs to D_{2h}^{16}, the four sulphur atoms in the unit cell must occupy one position 4 (c), as a sulphate group has no centre of symmetry, which is required in the positions 4 (a) and 4 (b). (As for the point positions 4 (a), 4 (b), and 4 (c) see page 538.) Moreover, if a sulphate group is situated between two symmetry planes, they should be at least 4.3_0 Å apart, since otherwise the distance between two equivalent oxygen atoms (belonging to different sulphate groups) would be too short. In $Th(OH)_2SO_4$, however, the distance between the symmetry planes $y = \frac{1}{4}$ and $y = -\frac{1}{4}$ is only 3.0_2 Å, and this fact, too, indicates that the sulphur atoms are situated in 4 (c), that is in the symmetry planes.

However, if a sulphur atom is situated in a symmetry plane, the four oxygen atoms belonging to it must be arranged in the following way: two oxygen atoms in the symmetry plane, one 1.2_0 Å below it and one 1.2_0 Å above it, the line between the centres of the last two atoms being perpendicular to the plane. Thus the atoms belonging to the four sulphate groups in the unit cell occupy the following point positions:

4 S in 4 (c)
4 O_1 in 4 (c)
4 O_2 in 4 (c)
8 O_3 in 8 (d) with $y = 0.05$.

In order to find out the x and z coordinates of these atoms the sections $y = 0.05$ and $y = 0.25$ in the unit cell were drawn, and the "forbidden areas" around symmetry centres and atoms already located were marked out (Fig. 3 a and b). As is seen in Fig. 3 b, the two oxygen atoms O_3 with $y = 0.05$ (belonging to the two sulphate groups at $y = \frac{1}{4}$) can be situated either one in the area P and one in P_1, or one in Q and one in Q_1. If they were situated in P and P_1, at most the following oxygen atoms from a sulphate group with its sulphur atom at $y = \frac{1}{4}$ could be in contact with a thorium atom (*cf.* Fig. 3: thick circles indicate Th—O contacts.):

$$\left.\begin{array}{l} 1\ O_3 \text{ with } y = 0.05. \\ 1\ O_3 \text{ with } y = 0.45. \end{array}\right\} \text{(Fig. 3 b)}$$

1 O_1 or O_2 with $y = \frac{1}{4}$. (Fig. 3 a).

The coordination number of thorium would then be 7. If they are situated in Q and Q_1, however, all the sulphate oxygen atoms can be in contact with thorium atoms. In this case every thorium atom is surrounded by 8 oxygen atoms. As the coordination number of thorium is 8 in most of its compounds, it seems probable that the oxygen atoms in question are situated in Q and Q_1 rather than in P and P_1.

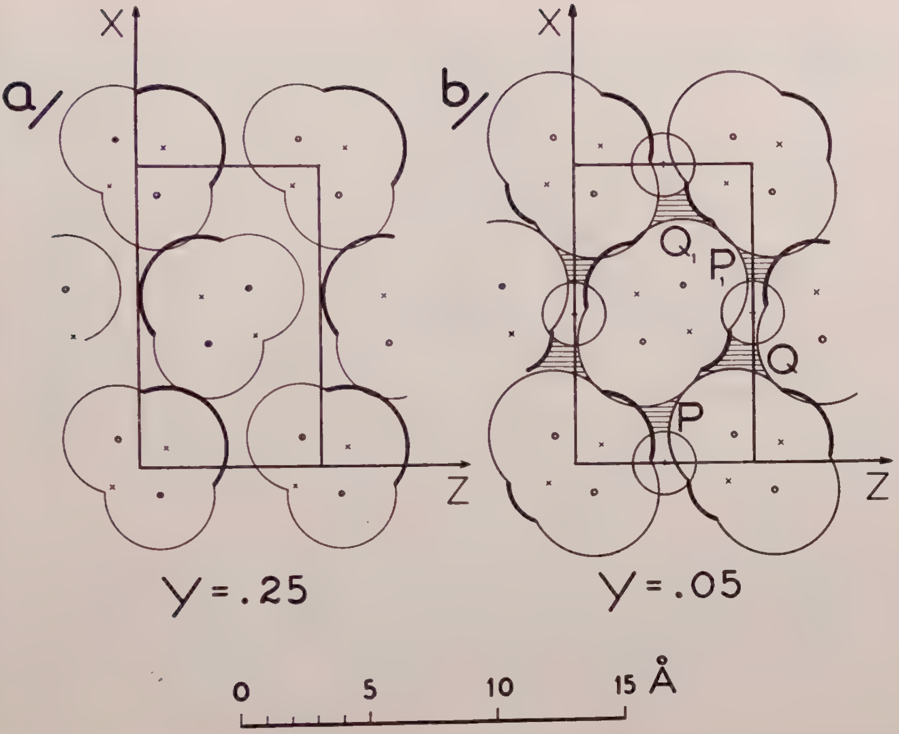

Fig. 3. Sections at a) $y = 0.25$ and b) $y = 0.05$ through the unit cell, showing the areas forbidden for the centres of sulphate oxygen atoms. As for the shaded areas P, Q, P_1, and Q_1, see the text page 542. Thick circles indicate Th — O contacts and thin circles O — O contacts. The relative size of a sulphate group is seen in Fig. 4.
× = (projection of) Th centre o = (projection of) O centre.

In order to determine the position of the sulphate group having one of its oxygen atoms in Q, an enlarged picture of this area was drawn (Fig. 4). In this figure continuous lines indicate restrictions in the plane $y = 0.25$ and dotted lines restrictions in $y = 0.05$. Thick lines indicate Th—O contacts. As was mentioned above, every one of the oxygen atoms belonging to the sulphate group should be in contact with a thorium atom. This can only occur if one atom (O_3) is situated somewhere on the arc AB, one (O_1) on CD and one (O_2) on EF. But whereever O_2 is situated on EF, O_1 is close to the point S on CD, if the distance $O_1 — O_2$ is 2.4_0 Å. On the other hand, if O_1 is situated at S, O_3 on AB must be at R and consequently O_2 at T on EF. The coordinates of the sulphate oxygen atoms would then be:

4 O_1 in 4 (c): $x = 0.47$, $z = 0.05$.
4 O_2 in 4 (c): $x = 0.27$, $z = 0.09$.
8 O_3 in 8 (d): $x = 0.35$, $y = 0.05$, $z = 0.82$.

Finally, the coordinates of the sulphur atoms, being situated at the centres of the SO_4 tetrahedra, follow immediately from the oxygen parameters above:

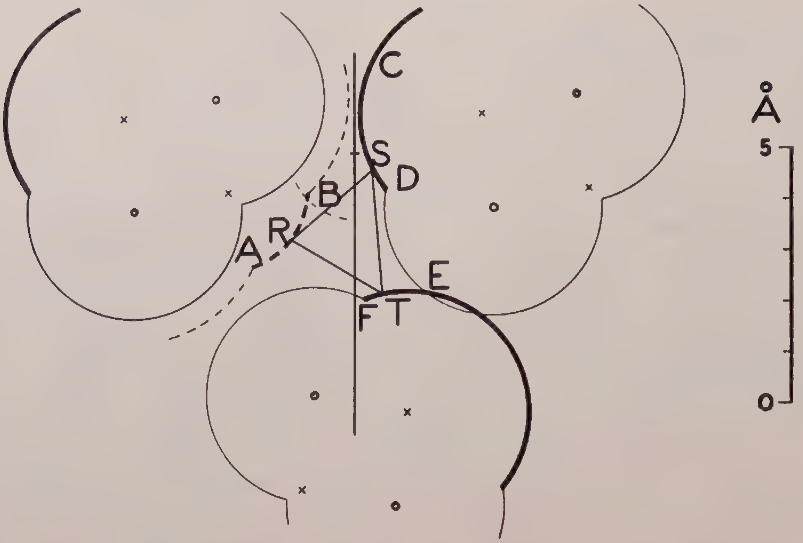

Fig. 4. Parts of the sections at $y = 0.25$ (continuous lines) and $y = 0.05$ (dotted lines) through the unit cell. The projection of the sulphate group having one of its oxygen atoms in Q (Fig. 3) is shown. As for the arcs AB, CD, and EF and the points R, S, and T see the text, page 543.
× = (projection of) Th centre o = (projection of) O centre.

4 S in 4 (c): $x = 0.36$, $z = 0.95$.

The accuracy in the parameters for the sulphur and oxygen atoms is estimated to be about ± 0.03.

The hydrogen atoms must be situated at the oxygen atoms O_{Th} forming OH^- ions, as otherwise the structure would be built up of ThO_2 chains and neutral H_2SO_4 molecules, which does not seem very probable. As the OH^- ions formed are in contact with some oxygen atoms from sulphate groups (*cf.* page 547), the hydrogen atoms surely form hydrogen bonds between O_{Th} and one of the neighbouring oxygen atoms.

Final proposed structure

After the positions of the sulphur and oxygen atoms had been found, the coordinates of the thorium atoms were redetermined more accurately from the intensities of the $h0l$, taking into account the scattering power of the light atoms (with the exception of H). The best agreement between observed and calculated intensities was then obtained, when thorium was situated in 4 (c) with $x = 0.064 \pm 0.003$ and $z = 0.148 \pm 0.005$. As the change in coordinates for the thorium atoms was so small, no redetermination of the oxygen and sulphur parameters was necessary.

In Tables 2 and 3 the calculated values of $100\left(\frac{F}{4\,f_{Th}}\right)^2$ are compared with the observed intensities of the reflections in the Weissenberg photographs $h0l$ and

Table 2

Calculated and observed intensities $h0l$ from a Weissenberg photograph of $Th(OH)_2SO_4$

Cu Kα-radiation.

$$I_{\mathrm{calc}} = 100\left(\frac{F}{4\,f_{\mathrm{Th}}}\right)^2$$

l ↓ \ h →	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	—	—	47	—	8	—	49	—	67	—	59	—	1	—	80
			m		vw$^-$		w		w+		w		—		m$^-$
1	—	20	41	10	42	16	20	55	1	21	38	0	87	3	27
		st	st	vw	vw	vvw	vw$^-$	w	—	vw	vw	—	w+	—	vw
2	3	4	2	31	0	120	4	15	14	23	2	78	1	66	4
	vvw	vw$^-$	vvw	m$^-$	—	m$^-$	—	vvw$^-$	vvw	vw	—	w	—	w+	—
3	—	85	2	18	6	21	2	83	1	61	7	7	22	14	12
		st	vvw	w$^-$	vvw$^-$	vw$^-$	—	vvw	—	vw+	—	—	vvw	vvw	vw
4	90	4	31	35	0	17	55	0	69	6	20	25	2	32	
	m+	vvw	w$^-$	w	—	vvw	vw$^-$	—	vvw	—	vvw	vvw	—	vw	
5	—	3	41	0	104	2	67	0	1	0	64	0	84		
		—	w	—	w+	—	vvw	—	—	—	w	—	w+		
6	49	12	27	34	0	8	23	5	64	2	31	58			
	w	vvw	vw	vw	—	—	—	—	vvw	—	vw	w			
7	—	96	17	8	2	18	0	72	0	70					
		w	—	—	—	—	—	vw	—	w+					
8	6	10	10	72	0	70									
	—	—	—	vvw	—	vvw									

$h1l$. Because of the shape of the crystals (thin plates) there is an absorption effect in these photographs (*cf.* WELLS 1937), and this effect is very pronounced for rays passing along the crystal plate. The line along which this high absorption occurs is indicated in the tables. Reflections on either side of this line must not be compared with each other. If this absorption and the general weakening of the reflections with increasing θ are taken into account, the agreement between observed and calculated intensities is rather good.

Thus the following structure is proposed for $Th(OH)_2SO_4$:

$D_{2h}^{16} - Pnma$, 4 formula units in the unit cell.

	x	y	z
4 Th in 4 (c)	0.064±0.003	0.25	0.148±0.005
4 S in 4 (c)	0.36	0.25	0.95
8 O_{Th}(OH) in 8 (d)	0.40	0±0.04	0.39
4 O_1 in 4 (c)	0.47	0.25	0.05
4 O_2 in 4 (c)	0.27	0.25	0.09
8 O_3 in 8 (d)	0.35	0.05	0.82

$4\,(c) = \pm(x, \tfrac{1}{4}, z),\ \pm(\tfrac{1}{2}+x, \tfrac{1}{4}, \tfrac{1}{2}-z)$

$8\,(d) = \pm(x, y, z),\ \pm(\tfrac{1}{2}+x, \tfrac{1}{2}-y, \tfrac{1}{2}-z),\ \pm(\bar{x}, \tfrac{1}{2}+y, \bar{z}),\ \pm(\tfrac{1}{2}-x, \bar{y}, \tfrac{1}{2}+z).$

The accuracy of the sulphur and oxygen parameters is estimated to be about ± 0.03.

Table 3

Calculated and observed intensities $h\,1\,l$ from a Weissenberg photograph of $Th(OH)_2SO_4$

Cu Kα-radiation.

$$I_{\text{calc}} = 100\left(\frac{F}{4\,f_{\text{Th}}}\right)^2$$

l ↓ \ h →	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	—	—	24 w	—	110 m	—	51 w+	—	4 —	—	87 w+	—	71 w+	—	43 w+
1	63 vst	19 m+	31 m	36 w+	1 —	25 vw	44 w	4 —	67 w+	1 —	21 vw	35 vw	1 —	37 w	31 w+
2	—	79 st	9 w	6 vw	8 vw	23 vw	3 —	61 w	0 —	83 w+	1 —	10 vvw	14 vvw	25 vw	
3	14 w	12 w	5 vw	64 m	3 —	74 w+	11 vvw	8 —	15 vvw	26 vw$^-$	3 —	85 w	0 —	60 w+	
4	—	26 w	40 w+	9 vvw	74 w+	2 —	27 vvw	41 vvw	0 —	22 vvw	41 vw	1 —	69 w+		
5	110 m$^-$	0 —	41 w	0 —	4 —	1 —	49 vw	0 —	85 vw	1 —	52 w	0 —			
6	—	29 vw	34 vw	16 vvw	55 vw	5 —	28 vvw	53 vvw	0 —	34 w$^-$	27 w$^-$				
7	1 —	8 —	6 —	76 vw	1 —	81 vw	4 —	10 —	10 —	33 w					
8	—	78 vw	16 —	4 —											

Description of the structure

The structure proposed for $Th(OH)_2SO_4$ is closely related to that of $Th(OH)_2CrO_4H_2O$ (LUNDGREN and SILLÉN 1949), since both of them contain long strings of the composition $[Th(OH)_2]_n^{2n+}$ (Fig. 2), formed by two almost parallel rows of OH^- ions held together by thorium atoms situated on alternate sides of the OH^- rows. The chains run in the direction of the y axis, which has about the same length in both structures (6.02_8 kX and 6.10_1 kX). In $Th(OH)_2SO_4$ they are held together by sulphate groups, each of which is in contact with three different chains.

The thorium atoms are surrounded by 8 oxygen atoms (4 hydroxide and 4 sulphate oxygen atoms) forming two almost parallel, somewhat distorted squares turned at an angle of 45° to each other, which configuration is also found in $Th(OH)_2CrO_4H_2O$. As pointed out by PAULING (1945), the coordination polyhedron formed in this way, the square Archimedean antiprism, gives a more satisfactory ionic coordination than the cube.

The xz and xy projections of the structure are shown in Fig. 5 and 6.

The distances Th—O, S—O, and O—O (within a sulphate group) were assumed to be about 2.4_0 Å, 1.4_7 Å, and 2.4_0 Å. The distances between oxygen atoms not belonging to the same sulphate group would be (in Å):

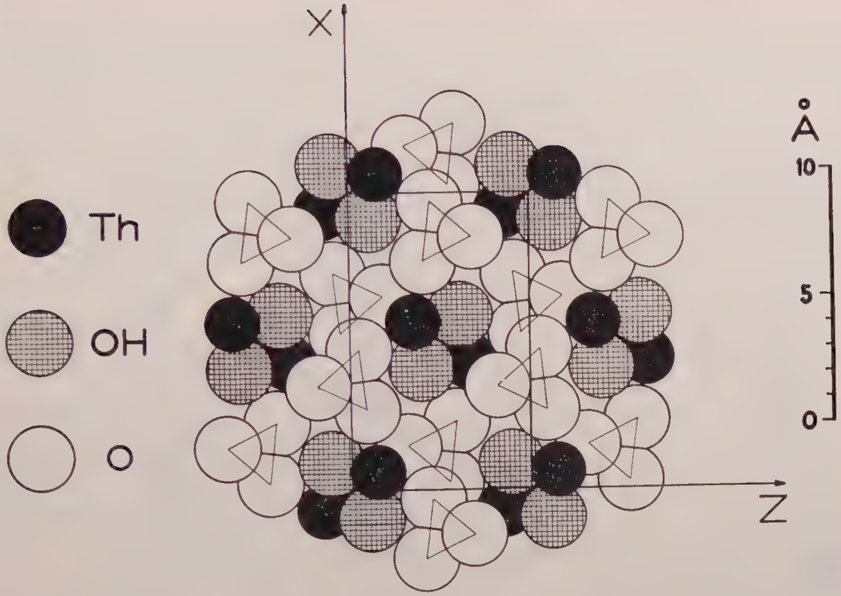

Fig. 5. Projection on the xz plane of the structure of $Th(OH)_2SO_4$. The size of a unit cell is indicated. The $[Th(OH)_2]_n^{2n+}$ chains are seen from above. Each SO_4^{2-} tetrahedron looks like a triangle, as two of its oxygen atoms coincide in this projection.

$$O_{Th} - 3\ O_{Th} = 2.8_2,\ 2 \times 3.0_2$$

$$O_{Th} - O_1,\ O_1 - 2\ O_{Th} = 2.9_6$$

$$O_{Th} - 2\ O_2,\ O_2 - 4\ O_{Th} = 2.8_8,\ 3.0_2$$

$$O_{Th} - 3\ O_3,\ O_3 - 3\ O_{Th} = 2.9_9,\ 3.1_1,\ 3.3_0$$

$$O_1 - 2\ O_1 = 2 \times 3.1_9$$

$$O_1 - 2\ O_3,\ O_3 - O_1 = 2 \times 2.9_4$$

$$O_2 - 2\ O_3,\ O_3 - O_2 = 2 \times 2.8_1$$

These figures are all within the normal range, showing that the lattice is also supported by O — O contacts. However, because of the difficulty in determining the oxygen parameters they may be inaccurate to within a few 0.1 Å.

With the structure proposed above a small hole is left in the plane $y = \frac{1}{4}$. The lattice seems, however, to be rather stable, as every oxygen atom is in contact with 9—11 neigbouring atoms, which is not far from 12, the number of neighbours in the closest packing of equivalent spheres.

I wish to thank Professor LARS GUNNAR SILLÉN for having suggested this work and for the very great interest he has shown in it and for the many valuable discussions I have had with him.

My thanks are due to Professor GUSTAF LJUNGGREN and Professor ARNE

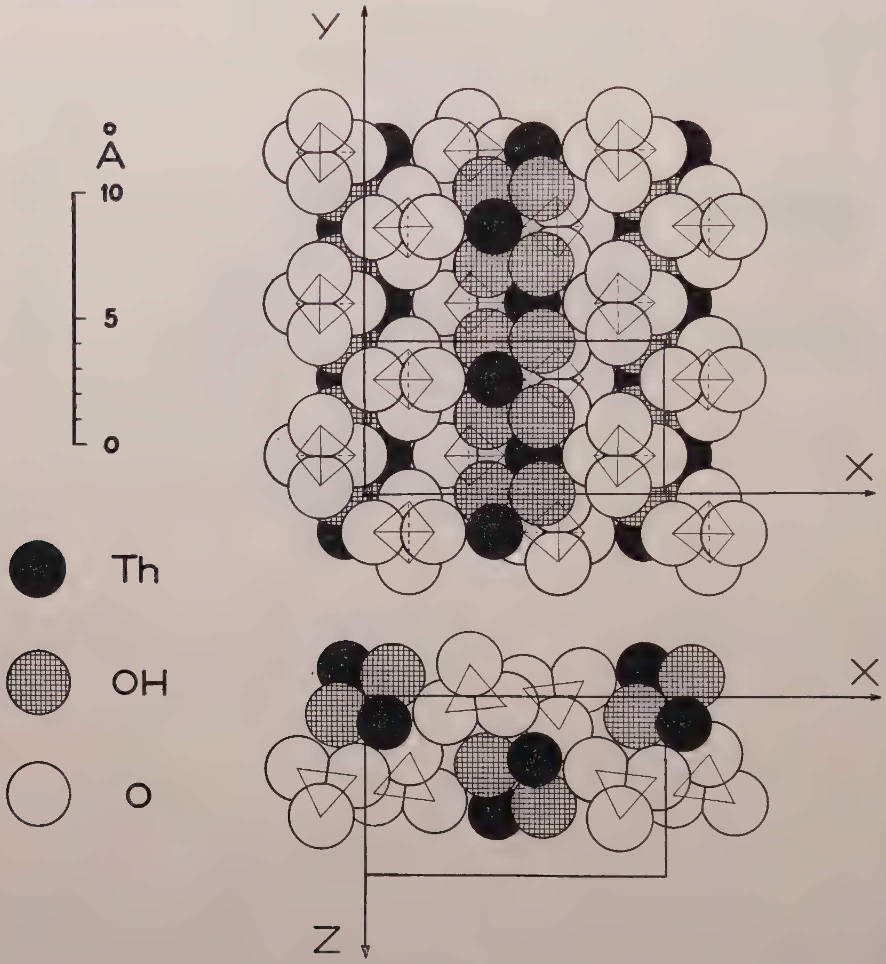

Fig. 6. Projection on the xy plane of the structure of Th(OH)$_2$SO$_4$. Part of the xz projection is drawn below. The $[\mathrm{Th(OH)_2}]_n^{2n+}$ chains run in the direction of the y axis.

ÖLANDER for their kind interest and for having provided me with laboratory facilities.

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Mr. SVEN SAHLQVIST has been an invaluable aid in the X-ray work.

SUMMARY

Th(OH)$_2$SO$_4$ has a primitive orthorhombic cell with the dimensions:

$$a = (11.709 \pm 0.005)\ \mathrm{kX} = (11.733 \pm 0.005)\ \text{Å}$$
$$b = (6.028 \pm 0.005)\ \mathrm{kX} = (6.040 \pm 0.005)\ \text{Å}$$
$$c = (7.045 \pm 0.005)\ \mathrm{kX} = (7.059 \pm 0.005)\ \text{Å}$$
$$V = 497.2_5\ \mathrm{kX}^3 = 500.2_7\ \text{Å}^3.$$

4 formula units in the unit cell.

A structure is proposed with atomic positions in D_{2h}^{16} (see page 545). The parameters of the thorium atoms were determined from the intensities. The positions of the light atoms were deduced from geometrical considerations, when the interatomic distances were assumed to be not far from those found in previously determined crystal structures. With these assumptions the structure given seems to be the only plausible one. The crystals are built up of long zigzag formed chains of composition $[Th(OH)_2]_n^{2n+}$ (Fig. 2) held together by sulphate groups. A comparison is made with the crystal structure of $Th(OH)_2CrO_4H_2O$.

Försvarets forskningsanstalt, Avd. 1.
Institute of Inorganic and Physical Chemistry, University, Stockholm.

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Über den Feinbau der Viscoseseide

Von Edgar Ribi

Mit 9 Figuren im Text

Einleitung

Die Viscoseseide wird seit vielen Jahren zur Ergründung ihres Feinbaus eingehend untersucht.

Während über den Aufbau der nativen Zellulosefasern eine allgemein gültige Vorstellung besteht, gehen die Meinungen hinsichtlich des genaueren Bauprinzipes bei der regenerierten Zellulose noch auseinander. In diesem Zusammenhang soll auf die Bemühungen von Kratky[1] und Hermans[2] hingewiesen werden, welche die verschiedenen Anschauungen einander gegenübergestellt und objektiv diskutiert haben.

Einen wertvollen Einblick in den Feinbau der regenerierten Zellulose erlauben die zahlreichen Hydrolyseversuche mit verdünnten Säuren, wobei der heterogene Reaktionsverlauf in engen Zusammenhang mit dem anatomischen Bau der Faser gebracht werden kann. Dasselbe gilt natürlich auch für die nativen Zellulosen. Staudinger und Sorkin[3] haben gezeigt, dass der Abbau der Zellulosefasern bei der Säurebehandlung anfänglich rasch erfolgt, sich später wesentlich verlangsamt und schliesslich praktisch zum Stehen kommt, d. h. offenbar ein GrenzDP für die Zelluloseketten erreicht wird. Die kinetische Verfolgung des hydrolytischen Abbaus, kombiniert mit Feuchtigkeitssorptionsmessungen und einigen röntgenographischen Untersuchungen erlaubten später Nickerson und Habrle[4] die Annahme, dass anfänglich die am wenigsten widerstandsfähigen Partien der Zellulosestruktur abgebaut werden, welche willkürlicherweise als amorph-mesomorphe Phase bezeichnet wurde. Es kommt dabei zur Anreicherung des resistenteren kristallinen Anteils. Die Hydrolysemethode, in der Folge von mehreren Forschern wertvoll erweitert[5], gestattete auch quantitative Aussagen über das Verhältnis zwischen leicht zugänglichen und kristallinen Mengen. Danach nimmt der Anteil an amorphem und mesomorphem Material in der Reihenfolge Baumwolle, Holzzellulose, mercerisierte Baumwolle und mercerisierte Holzzellulose zu und ist am grössten für Viscoseseide. In der gleichen Folge wurde eine entsprechende Verkleinerung der viskosimetrisch ermittelten Längen der Zellulosemoleküle der gelösten Hydrolyserückstande festgestellt und dieses Ergebnis mit der Abnahme der Längen der kristallinen Bereiche (Mizellen) in Zusammenhang gebracht. Die Mizellen in der Viscoseseide müssten danach am kürzesten sein. Zu übereinstimmenden Resultaten gelangten METHA und PACSU[1] auf Grund genauerer viskosimetrischer Polymolekularitätsmessungen an hydrolysierter Viscoseseide.

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[1] O. Kratky, Z. angew. Chem. *53*, 153 (1940).

[2] P. H. Hermans, „Physics and Chemistry of Cellulose Fibres“, Amsterdam 1950.

[3] H. Staudinger und M. Sorkin, Ber. *70*, 1565 (1937).

[4] R. F. Nickerson, Ind. Eng. Chem. Anal. Ed. *13*, 423 (1941); Ind. Eng. Chem. *33*, 1022 (1941); *34*, 85, 1480 (1942).
R. F. Nickerson und J. A. Habrle, Ind. Eng. Chem. *37*, 1115 (1945); *39*, 1507 (1947)

[5] G. Goldfinger, H. Mark und S. Siggia, Ind. Eng. Chem. *35*, 1083 (1943);
C. M. Conrad und A. G. Scroggie, Ind. Eng. Chem. *37*, 542 (1945);
H. J. Philipp, M. L. Nelson und H. M. Ziifle, Text. Res. J. *11*, 585 (1947);
M. L. Nelson und C. M. Conrad, Text. Res. J. 149 (1948).

Angaben über Existenz und Grösse der Mizellen in der Viscoseseide stammen schon aus dem Jahre 1928 von HENGSTENBERG und MARK[2]. Aus den Breiten der Röntgeninterferenzen wurde auf eine Länge von ungefähr 300 Å und eine Dicke von rund 40 Å geschlossen. Später fanden FANKUCHEN und MARK[3] mit Hilfe der röntgenographischen Kleinwinkelstreumethode eine mittlere Mizellbreite von 50—200 Å.

Quantitative Aussagen über den Kristallinitätsgrad der verschiedenen Fasern erlaubten auch Intensitätsmessungen an Röntgendiagrammen[4 5 6], Dichtemessungen[4], Feuchtigkeitssorptionsversuche[4 7 8] und die Methode des Deuteriumaustausches[9]. Die Resultate dieser Methoden stehen im Einklang mit der von Nickerson und Habrle mitgeteilten Gliederung der verschiedenen Zellulosen nach ihrem Kristallinitätsgrad. Ein genauerer Vergleich zwischen den Ergebnissen der Hydrolyseversuche und denjenigen der physikalischen Methoden zeigt aber eine Diskrepanz die mit einer Rekristallisationserscheinung bei der Säurebehandlung in Zusammenhang gebracht werden kann und welche für die regenerierte Zellulose am ausgeprägtesten sein muss[8 10 11].

Svedberg und seine Mitarbeiter haben seit vielen Jahren die Zellulose zur Ergründung ihrer Polymolekularität und Feinstruktur untersucht. Zusammengefasste Ergebnisse hat SVEDBERG[12] laufend in Vorträgen mitgeteilt. RÅNBY[13] führt am hiesigen Institut eingehende Hydrolyseversuche an verschiedenen Zellulosefasern aus und ermittelt die Polymolekularität der nitrierten Depolymerisationsprodukte. Zur morphologischen und strukturellen Identifizierung der Hydrolyseprodukte nativer und mercerisierter Zellulosen haben RÅNBY[14] und RIBI[11] elektronenoptische und röntgenographische methoden herangezogen.

Auf Grund mikroskopischer Ergebnisse wurde gezeigt, dass die mittlere Kristallitlänge in Einklang mit den erhaltenen Polymolekularitätsmessungen gebracht werden kann. Ferner führte die Mikroskopierung einer mit Ultraschall behandelten Baumwollfaser zur Annahme, dass die Mizellen in der nativen Zellulose zu einheitlichen Elementarfibrillen verknüpft vorliegen müssen.

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[1] P. C. METHA und E. PACSU, Text. Res. J. *19*, 625, 699 (1949).

[2] J. HENGSTENBERG und H. MARK, Z. Krist. *69*, 271 (1928).

[3] I. FANKUCHEN und H. MARK, J. Applied Phys. *15*, 364 (1944).

[4] P. H. HERMANS, „Contribution to the Physics of Cellulose Fibres", Amsterdam 1946.

[5] H. G. INGERSOLL, J. Applied Phys. *17*, 924 (1946).

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[7] P. H. HERMANS und A. WEIDINGER, J. Am. Chem. Soc. *68*, 1138 (1946).

[8] J. A. HOWSMON, Text. Res J. *19*, 152 (1949).

[9] V. J. FRILETTE, I. HANLEY und H. MARK, J. Am. Chem. Soc. *70*, 1107 (1948).

[10] F. C. BRENNER, V. J. FRILETTE und H. MARK, J. Am. Chem. Soc. *70*, 877 (1948).

[11] B. G. RÅNBY und E. RIBI, Experientia *6*, 12 (1950).

[12] T. SVEDBERG, Svensk Papperstidning *45*, 444 (1942); Cellulosechemie *21*, 57 (1943); J. Phys. Coll. Chem. *51*, 1 (1947); Svensk Papperstidning 52, 157 (1949).

[13] Referiert von P. O. KINELL und B. G. RÅNBY in: Advances in Colloid Science Vol III, im Druck (New York).

[14] B. G. RÅNBY, Acta Chemica Scandinavica *3*, 649 (1949).

Analoge Untersuchungen sollten an regenerierter Zellulose vorgenommen werden, um über deren Feinbau näheres zu erfahren. Erste Resultate sind bereits von Rånby und Ribi[1] mitgeteilt worden. Danach liess die Röntgenanalyse vermuten, dass nach der Depolymerisation von Viscoseseide mit verdünnter Mineralsäure Kristallite mit extrem kleinen Dimensionen angereichert werden. B. G. Rånby hat an den gelösten Hydroloyseprodukten der Viscoseseiden Polymolekularitätsmessungen ausgeführt und wird hierüber an anderer Stelle berichten. Hier soll über die weitergeführten morphologischen und strukturellen Studien mitgeteilt werden.

Experimente und Resultate

Folgende Viscoseseiden gelangten zur Untersuchung:

Bezeichnung	Herkunft	Ausgangsmaterial	Streckung
V_{II}	N.V. Onderzoekningsinstitut Res. Arnhem, Holland.	Borregard S.V.S.	15 %
V_{III}	»	50 % Linters, 50 % Cordicel	70 %
V_{IV}	Mo och Domsjö AB, Schweden.	Holzzellulose*	1 %
V_{V}	»	Holzzellulose*	58 %
V_{VI}	»	Holzzellulose*	Isotrope Fiber

* Sulfitzellulose, α-Gehalt 95,3 %.

Zur Reinigung wurden die Seiden mit Aether-Benzol-Alkohol im Soxlet behandelt.

Viscoseseiden mit Ultraschall behandelt

Die Mikroskopierung der bei nicht sehr grosser Schallstärke behandelten Seiden zeigt, dass ein Zerfall in Fibrillen sehr einheitlicher Dicke stattgefunden hat, welche derjenigen der Elementarfibrillen der nativen Zellulose entspricht (Fig. 3 u. 4, Tab. 2). Eine vollständige Dispergierung in solche Stränge konnte an Viscoseseiden verschiedener Herkunft, ohne und unter grosser Streckung gesponnen, beobachtet werden. Diese Fibrillen, welche als Mizellarstränge der regenerierten Zellulose aufzufassen sind, können mit Längen bis zu mehreren μ auftreten.

Teilweise werden die Strangbündel von Gelhäuten überzogen, deren Elektronendurchlässigkeit kleiner ist als diejenige des Zellulosematerials. Neben dieser amorphen Ausbildungsform sind auch vereinzelt hexagonal-blättchenförmige Kristalle mit Linearausdehnungen von einigen tausend Å zu erkennen. Es wird angenommen, dass es sich bei dieser Begleitkomponente um eine Zink-Hydroxyverbindung handelt, welche sich beim Fällen der alkalischen Viscose im sauren zinksalzhaltigen Bade bilden konnte. Hexagonal begrenzte, laminare Kristalle kolloider Dimensionen sind bekanntlich charakteristisch für viele von Feitknecht und Mitarbeitern untersuchte Hydroxyde und basische Salze zweiwertiger Metalle.[2] Dass der formlose Anteil ausser amorpher Zellulose, auch aus der Zinkverbindung bestehend angenommen werden kann, ergibt sich aus der Beobachtung, dass zwischen den gelartigen Ausbildungsformen und den wohlausgebildeten Kristallen alle möglichen Übergangsformen auftreten, vollständig analog wie sich dies bei der elektronenmikroskopischen Untersuchung von Mangan-Hydroxychloriden feststellen liess.[1]

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[1] B. G. Rånby und E. Ribi, Experientia *6*, 12 (1950).
[2] W. Feitknecht, Koll. Z. *92*, 257 (1940).

Ferner gelangte eine isotrope Modellfiber zur Untersuchung. Eine solche Fiber wird bekanntlich durch Fällen der Viscose unter möglichster Verhinderung einer Orientierung der Mizellen erhalten. Nach der Dispergierung mit Ultraschall liegt ein sehr polydisperses Material vor. Es zeigen sich kleinste Partikeln von ungefähr 100 Å Grösse, Stäbchen und Fäserchen mit Längen von einigen tausend Å, deren recht einheitliche Dicke 50—100 Å beträgt. Vereinzelt sind auch grosse mizellarstrangartige Aggregate festzustellen (Fig. 5). Das Debye-Diagramm der isotropen Fiber unterschied sich nicht von demjenigen einer desorientierten kommerziellen Viscoseseide. Solche Ausbildungsformen dürften in einer von KRATKY[2] und HERMANS[3] vorgeschlagenen Anordnung (Ordnung in kleinen Bereichen) der isotropen Fiber eigen sein.

Mit Säure behandelte Viscoseseiden

Der hydrolytische Abbau der Seiden erfolgte ausschliesslich in siedender 2,5 n Schwefelsäure. Nach der Einwirkung der verdünnten Mineralsäure tritt eine Verbesserung der Pulverdiagramme ein. Die Intensität der Interferenzen wird grösser und die Untergrundschwärzung nimmt ab (Fig. 1). Eine wesentliche Verbesserung der Röntgenaufnahmen tritt schon nach einer Säurebehandlung von zwei Minuten ein. Die Breite der Interferenzen ist aber recht beträchtlich. Als Ursache kommt kaum ein stärker fehlgeordnetes Gitter in Frage, vielmehr ist hierfür die Kleinheit der bei der Hydrolyse angereicherten kristallinen Teilchen verantwortlich zu machen. Es lassen sich nämlich linienreiche Elektronenbeugungsdiagramme herstellen, deren Interferenzen nur eine geringe Unschärfe zeigen (Fig. 6). Auf Grund des Vergleiches zwischen Röntgen- und Elektronenbeugungsaufnahmen kann qualitativ eine mittlere Teilchengrösse von ungefähr 100 Å angenommen werden.

Die frühere Elektronenmikroskopierung hydrolysierter mercerisierter Zellulose zeigte Teilchen mit einer mittleren Länge von einigen hundert Å und einheitlicher Dicke, zwischen 50–100 Å liegend.[4]

Vergleicht man nun das Röntgendiagramm der hydrolysierten Viscoseseiden mit demjenigen der hydrolysierten mercerisierten Zellulose, so fällt eine selektive Verschiedenheit der Interferenzintensitäten auf, welche Aufschluss über die unterschiedliche Ausbildungsform der beiden kolloiden Hydrolyseprodukte zu geben vermag (Fig. 2). Während die Basisinterferenz 002 auf den beiden Diagrammen analog ausgebildet erscheint, ist der Prismenreflex 020 der hydrolysierten Viscoseseide nur sehr schwach und verbreitert angedeutet. Bei der mercerisierten Zellulose ist diese Linie weit intensiver und schmäler. Da bekanntlich die Zahl hintereinanderliegender Netzebenen umgekehrt proportional der Interferenzbreite ist darf man annehmen, dass die Länge der Kristallite der Viscoseseide kleiner sein

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[1] E. RIBI, Diss., Bern 1948.

[2] O. KRATKY, Kolloid-Z. *68*, 347 (1934).

[3] P. H. HERMANS, Kolloid-Z. *83*, 71 (1938).

[4] T. SVEDBERG, Svensk Papperstidning *52*, 157 (1949).

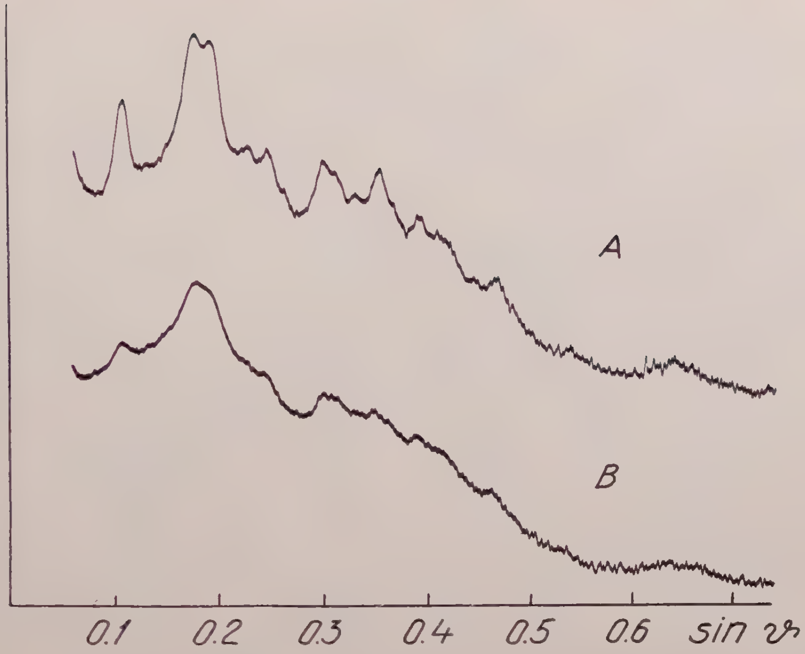

Fig. 1. Photometerkurven von Debye—Scherrer-Aufnahmen.

A) Hydrolysierte Viscoseseide (V_{II} 1 Std. mit 2,5 n H_2SO_4).
B) Viscoseseide (V_{II} desorientiert).

muss als diejenige der mercerisierten Zellulose, während man für deren Breite kaum einen Unterschied zu erwarten hat.

Beugungsversuche mit Elektronenstrahlen erwiesen sich infolge der Kleinheit der Kristallite als wertvolle Ergänzung der röntgenographischen Untersuchungen. Die grosse Intensität der Elektroneninterferenzen und deren Schärfe als Folge der kleineren Wellenlänge führen von den isolierten Kristalliten zu guten Durchstrahldiagrammen. Die Beugungsaufnahmen der verschiedenen Viscoseseiden, nach kürzerer und mehrstündiger Hydrolysedauer, zeigen weder Unterschiede in der Intensitätsverteilung noch in der Lage der Interferenzen.

Bei genauer Ausmessung der Diagramme mit Hilfe eines Mikrokomparators und zuverlässiger Ermittlung der Wellenlänge gelingt eine geeignete Auswertung.

Die beiden inneren intensiven Interferenzen der Diagramme der hydrolysierten Viscoseseide (Fig. 6, Tab. 1) stammen von der Netzebene $10\bar{1}$ bzw. 002.[1] Bei allen übrigen Linien fallen mehrere Interferenzen zusammen.

Die mittlere Streuung der beobachteten $\frac{\sin^2 \vartheta \cdot 4}{\lambda^2}$-Werte von $10\bar{1}$ und 002 betrug bei Berücksichtigung sämtlicher ausgemessenen Diagramme ± 0,0004, was einer Netzebenendifferenz von ungefähr ± 0,02 Å entspricht. Als Mittel für die Konstante der $10\bar{1}$ Linie wurde ein Wert

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[1] Eine innerste Interferenz, bei welcher es sich um 101 handeln dürfte, lässt sich nicht mehr genau vermessen, da diese zu nahe um den Durchstosspunkt und in der intensiven Primärschwärzung liegt.

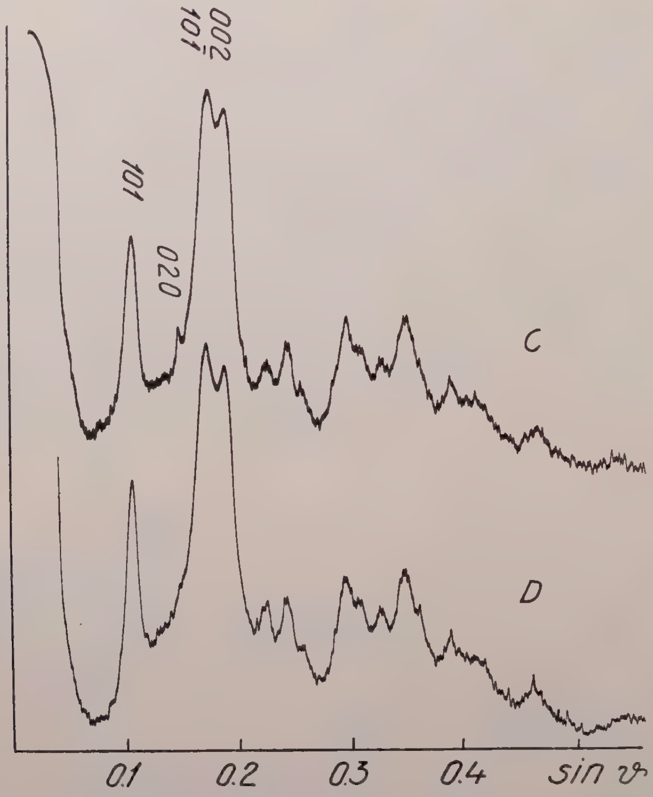

Fig. 2. Photometerkurven von Guinier-Pulveraufnahmen.

C) Hydrolysierte mercerisierte Holzzellulose (1 Std. mit 2,5 n H_2SO_4).
D) Hydrolysierte Viscoseseide (1 Std. mit 2,5 n H_2SO_4.

von 0,0511, für 002 einen solchen von 0,0613 erhalten, in guter Übereinstimmung mit den von Andress[1] berechneten Daten.

Die 0,151 entsprechende Linie der Beugungsdiagramme ist bei der mercerisierten Zellulose stets wesentlich intensiver als bei der regenerierten Zellulose. Unter dem zugehörigen Beugungswinkel ist die 040 Interferenz mit grösserer Intensität zu erwarten, allerdings vereint mit Reflexen anderer Netzebenen. Die selektive Verschiedenheit der Intensität dieser Linie kann daher auf die nach 040 grössere Ausdehnung bei den Kristalliten der mercerisierten Zellulose zurückgeführt werden.

Zur elektronenmikroskopischen Untersuchung wurden die Hydrolyseprodukte mit Wasser bis zur eintretenden Peptisation gewaschen. Die so erhaltenen getrübten Sole wurden direkt auf die Objektträger präpariert.

Im Einklang mit den Ergebnissen der Röntgen- und Elektroneninterferenzversuche konnten mikroskopisch Teilchen von globularer bzw. rotationsellipsoidarti-

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[1] R. K. Andress, Z. phys. Chem. Abt. B *4*, 190 (1929).

Tabelle 1

Auswertung der Röntgen- und Elektronenbeugungsdiagramme hydrolysierter Zellulosen.

Indizes	Faser	Kristallite				
	Faserdiagr.	Guinier-Pulver diagr.	Elektronenbeugungsdiagr.			
	Merc. Zellulose $\frac{\sin^2 \vartheta \cdot 4}{\lambda^2}$ ber.[1]	Merc. Zellulose hydrolysiert beob.	Merc. Zellulose hydrolysiert beob.	Int. gesch.	Viscoseseide (VIV) hydrolysiert (1 Std.) beob.	Int. gesch.
101	0,0185	0,0188	—	—	—	—
020	0,0374	0,0377	—	—	—	—
$10\bar{1}$	0,0511	0,0512	0,0513	sst	0,0511	sst
002	0,0616	0,0616	0,0613	st	0,0615	st
031	0,100	0,104	0,103	m	0,103	m
131	0,103					
130	0,104					
123	0,147	0,151	0,151	st	0,151	m
312	0,148					
013	0,148					
311	0,150					
$12\bar{2}$	0,151					
040	0,151					
$22\bar{1}$	0,163	0,166	0,166	s	0,166	s
230	0,163					
303	0,166					
041	0,166					
$14\bar{1}$	0,202	0,206	0,206	m	0,205	m
$20\bar{2}$	0,205					
103	0,207					

ger Gestalt beobachtet werden, mit Grössen im Bereiche von ungefähr 100 Å (Fig. 7 und 8).

Da sich die Kristallite scharf abbilden liessen, gelang eine genauere Ausmessung ihrer Grösse. Das Messen und die statistische Auswertung hat Fräulein ANNALISA NORLING ausgeführt.

Gemessen wurde an den Originalplatten (Mikroskopvergrösserung 8500 ± 2,5 %) mit Hilfe des Mikrokomparators. Für die Genauigkeit der wahren Teichenabmessungen darf eine Grössenordnung von ungefähr der Hälfte des Auflösungsvermögens des verwendeten Mikroskopes angenommen werden, d.i. schätzungsweise rund ±15 Å. Bei mehreren Komparatorablesungen an ein und demselben Teilchen entsprachen die grössten Messdifferenzen lediglich einigen wenigen Å-Einheiten.

In Tabelle 2 finden sich einige Resultate zusammengestellt. Für die grösste Linearabmessung der Kristallite, deren Länge, werden arithmetische Mittelwerte erhalten, welche zwischen 110 und 120 Å liegen und für die grösste Ausdehnung

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[1] R. K. ANDRESS, Z. phys. Chem. Abt. B *4*, 190 (1929).

Tabelle 2.

Ergebnisse der Teilchengrösseermittlung aus elektronenmikroskopischen Aufnahmen (nach A. NORLING).

Präparat	Elementarfibrillen		Kristallite*		
	n	Dicke in Å M	n	Dicke in Å M	Länge in Å M
Viscoseseide VII	80	79	300	74	116
Viscoseseide VIV	60	73	100	69	115
Viscoseseide VV	60	74	200	73	112
Isotrope Viscosefiber VVI	—	—	200	83	120
Baumwolle	40	74	—	—	—

* VII, VIV und VV nach 4 Std. mit 2,5 n H_2SO_4
VVI » 3 » » 2,5

n = Zahl der ausgemessenen Fibrillen und Kristallite
M = arithmetischer Mittelwert der Teilchendimensionen

senkrecht hierzu, sind die mittleren Dimensionen zwischen ungefähr 70 und 80 Å. Die Dicke der Kristallite stimmt auffallend mit derjenigen der Fibrillen überein.

Mit zunehmender Hydrolysedauer nimmt der peptisierbare Anteil beim Auswaschen der Hydrolyseprodukte zu. Die auch nach längerer Säurebehandlung nicht peptisierbaren Rückstande bestehen offenbar aus grösseren Kristallitaggregaten. Es kann nach kurzer Behandlung mit Ultraschall weiter peptisiert werden. Die Partikel dieser Sole führen zu normalen Beugungsdiagrammen und deren mikroskopische Beobachtung zeigt die charakteristischen Kristallite der Viscoseseide.

Seiden, während nur zwei Minuten mit verdünnter Schwefelsäure behandelt, liessen sich mit Ultraschall weitgehend bis zu den Kristallitdimensionen dispergieren (Fig. 9). Neben vereinzelten kürzeren Fäden, welche Mizellarstrangresten entsprechen, sind die Kristallite auch in Form von Bändern vereinigt zu erkennen. Letztere können als anhydrolysierte Mizellarstrangaggregate aufgefasst werden (vgl. Fig. 3 u. 4). Abbildung 9 mag daher den morphologischen Zusammenhang zwischen Kristallit und Elementarfibrille veranschaulichen.

Diskussion der Resultate

Im Zusammenhang mit den mitgeteilten Ergebnissen muss kurz auf die heute gültige Vorstellung über den Feinbau der nativen Zellulosefasern hingewiesen werden, welche sich vor allem aus den Arbeiten von STAUDINGER und SIGNER[1], FREY—WISSLING[2], KRATKY[3], MARK[4], SAUTER[5] und HERMANS[6] entwickelte. Es ist dies die neue Mizellartheorie, die Theorie vom makromolekular fibrillären Aufbau, welche aus der Erkenntnis Staudingers hervorging, wonach die Längen der Zellulosemoleküle wesentlich grösser sind, als die röntgenographisch ermittelte Länge der Zellulosemizellen. Man stellt sich die Bildung des Mizellargerüstes so vor, dass die langen Kettenmoleküle stellenweise zu gittermässig geordneten Bereichen (Mizellen) zusammentreten, wobei die Moleküle mehrere kristalline Bezirke durchziehen können. Auf diese Weise entstehen die aus „Fransenmizellen" aufgebauten Elementarfibrillen, deren Zusammenhalt dadurch erfolgt, dass Molekülfäden in benachbarte Stränge übergehen.

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[1] H. STAUDINGER und R. SIGNER, Z. Krist. *70*, 193 (1929).

[2] A. FREY—WISSLING, Protoplasma *25*, 261 (1936).

[3] O. KRATKY, Kolloid-Z. *70*, 14 (1935); *84*, 149 (1938).

[4] O. KRATKY und H. MARK, Z. phys. Chem. Abt. B *36*, 129 (1937).

[5] E. SAUTER, Z. phys. Chem. Abt. B *35*, 129 (1937).

[6] P. H. HERMANS, Kolloid-Z. *83*, 71 (1938).

Die Mikroskopierung der mit Ultraschall dispergierten Viscoseseiden hat gezeigt, dass auch hier ein Aufbau aus Elementarfibrillen vorliegt. Die Dicke der Fibrillen ist sehr einheitlich und ferner völlig analog derjenigen der nativen Faser.

Die röntgenographischen und elektronenoptischen Untersuchungen der Hydrolyseprodukte vermögen die in der Einleitung wiedergegebenen Annahmen von Nickerson und Habrle zu bestätigen. Eine Übereinstimmung hinsichtlich der mikroskopisch gefundenen Länge der Kristallite nach der Säurebehandlung von ungefähr 110-120 Å, besteht auch mit den viskosimetrischen Polymolekularitätsmessungen von Metha und Pacsu[1]. In der Folge werden auch entsprechende Messungen von B. G. Rånby und osmotische Bestimmungen der mittleren Grenz-DP-Werte von B. Enoksson einen weiteren Vergleich gestatten.

Trotz dieser anscheinend auffallenden Übereinstimmung darf betreffend den Absolutdimensionen der Kristallite auf Grund der mikroskopischen Ergebnisse, wie bereits erwähnt, keine genauere Aussage gemacht werden. Hingegen gestattet die Genauigkeit, mit welcher die mitgeteilten „Relativwerte" erhalten werden können, einen wertvollen Hinweis auf die Partikelform, deren Häufigkeitsverteilung und dem morphologischen Zusammenhang zwischen den isolierten Kristalliten und den Elementarfibrillen.

Über die Häufigkeitsverteilung der Kristallite bzw. diejenige der Linearabmessungen wird in einer nachfolgenden Arbeit berichtet, ergänzt mit Ergebnissen an weiteren Hydrolyseprodukten.

Die mittleren Längen der Zelluloseketten in den nichthydrolysierten Seiden betragen nach Angaben von B. Enoksson[2] grössenordnungsmässig ungefähr tausend Å. Diese Kettenlängen lassen sich mit den viel kleineren Kristallitlängen kaum anders in Zusammenhang bringen, als für die festen Seiden einen Aufbau aus gefransten Mizellen anzunehmen, derart, dass sich die Fadenmoleküle durch mehrere kristallisierte Bereiche und dazwischenliegende amorphe und mesomorphe Gebiete hindurch erstrecken. Es scheint also ein ganz ähnlicher Feinbau vorzuliegen, wie er für die native Zellulose beschrieben wurde. Ein Unterschied besteht offenbar in einer kleineren Länge der Mizellen und Fadenmoleküle und grösseren Anteilen an zwischenkristallinem amorph-mesomorphem Material bei der regenerierten Zellulose. Diese Strukturannahme spricht nicht gegen die bekannte Tatsache, wonach die Reissfestigkeit gegenüber der nativen Faser wenig verschieden, hingegen die Knickbruchfestigkeit bei der Viscoseseide grösser ist.

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[1] P. C. Metha und E. Pacsu (Text. Res. J. *19*, 625, 699 [1949]) fanden, dass ungefähr ein Drittel der Zelluloseketten der gelösten Hydrolyseprodukte mit einem einheitlichen DP von 62 vorliegt. Der Polymerisationsgrad wurde aus der Viskosität mit Hilfe der Formel von E. O. Kremer und W. D. Lansing errechnet (J. Phys. Chem. *39*, 153 [1935]). Die Auswertung nach der europäischen Schule Staudingers würde zu einem DP von 23 führen; dies entspricht einer Kettenlänge von ungefähr 120 Å.

[2] Nach vorläufigen osmotischen Messungen an gelösten Nitraten von Lic. B. Enoksson. Die genaueren Resultate sollen später in einem andern Zusammenhang mitgeteilt werden.

Aus den röntgenographischen Ergebnissen kann geschlossen werden, dass die Säurebehandlung der Viscoseseiden einen beträchtlichen Rekristallisationseffekt zur Folge hat, übereinstimmend mit den Aussagen von HOWSMON[1], HERMANS und WEIDINGER[2]. Zu dieser Annahme führt der Umstand, dass die Verbesserung der Diagramme nach erfolgtem Abbau der Faser bis zu den Kristallitdimensionen nicht im Einklang mit dem Substanzverlust zu bringen ist. Dass ferner die Rekristallisation bereits bei Beginn der Säurebehandlung eintritt, geht aus den Röntgenaufnahmen der während nur zwei Minuten in die heisse verdünnte Schwefelsäure eingetauchten Seiden hervor. Trotzdem hier der Substanzverlust noch sehr gering, ist die Diagrammverbesserung schon beträchtlich und die Verschiedenheit gegenüber dem Interferenzbild nach längerer Säurebehandlung ist nicht gross.

Diese Arbeit wurde unter der Leitung von Prof. THE SVEDBERG ausgeführt, welchem ich für sein reges Interesse und wertvolle Diskussionen herzlich danke.

Fil. lic. BENGT RÅNBY hat mich in seine Zelluloseuntersuchungen eingeführt. Ich danke ihm herzlich für die Zusammenarbeit, für seine grossen Bemühungen und dem Interesse welches er diesen Untersuchungen entgegenbrachte.

Prof. GUNNAR HÄGG hat mir ermöglicht in seinem Laboratorium Röntgenaufnahmen herzustellen. Ich danke ihm bestens, wie auch seinen Mitarbeitern, welche mir stets zuvorkommend die Arbeit erleichterten.

Dozent INGVAR JULLANDER und Dr. P. H. HERMANS möchte ich für die zur Verfügung gestellten Seideproben und ihr grosses Interesse meinen besten Dank aussprechen.

Die Auswertung der Beugungsdiagramme und Elektronenmikroskopaufnahmen hat in aufopfernder Weise Fräulein ANNALISA NORLING ausgeführt. Für die grosse Hilfe danke ich ihr herzlich.

Meinen besten Dank gilt Tekn. Lic. BERTIL ENOKSSON für die osmotischen Messungen, Fil. Lic. HÅKAN LEYON und Herrn GÖTE EKLUND für wertvolle Hilfe und Erleichterungen bei den elektronenmikroskopischen Arbeiten.

Schliesslich danke ich auch den schwedischen Zellulosefirmen Billeruds AB, Mo och Domsjö AB, Stora Kopparbergs Bergslags AB, Svenska Cellulosa AB und Uddeholms AB für die grosszügige finanzielle Unterstützung.

Uppsala, Physikalisch-chemisches Institut.

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[1] Leider ist dem Schreibenden die Arbeit von J. A. HOWSMON (Text. Res. J. *19*, 152 [1949]) erst nach Abschluss der hier mitgeteilten Ergebnisse bekannt geworden.

[2] Nach persönlicher Mitteilung und liebenswürdiger Überlassung des zum Druck eingereichten Manuskriptes der Arbeit über „Change in crystallinity upon heterogenous acid hydrolysis of cellulose fibres“.

Tryckt den 11 december 1950

Uppsala 1950. Almqvist & Wiksells Boktryckeri AB

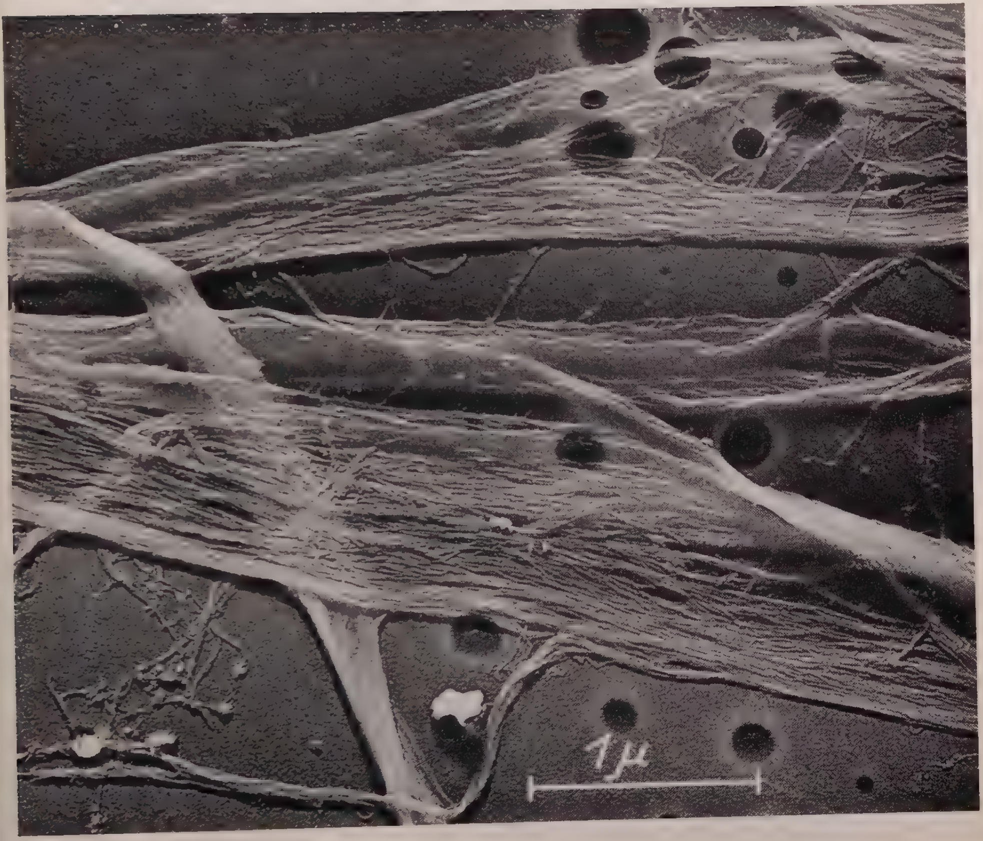

Fig. 3. Viscoseseide (VIV) mit Ultraschall behandelt.

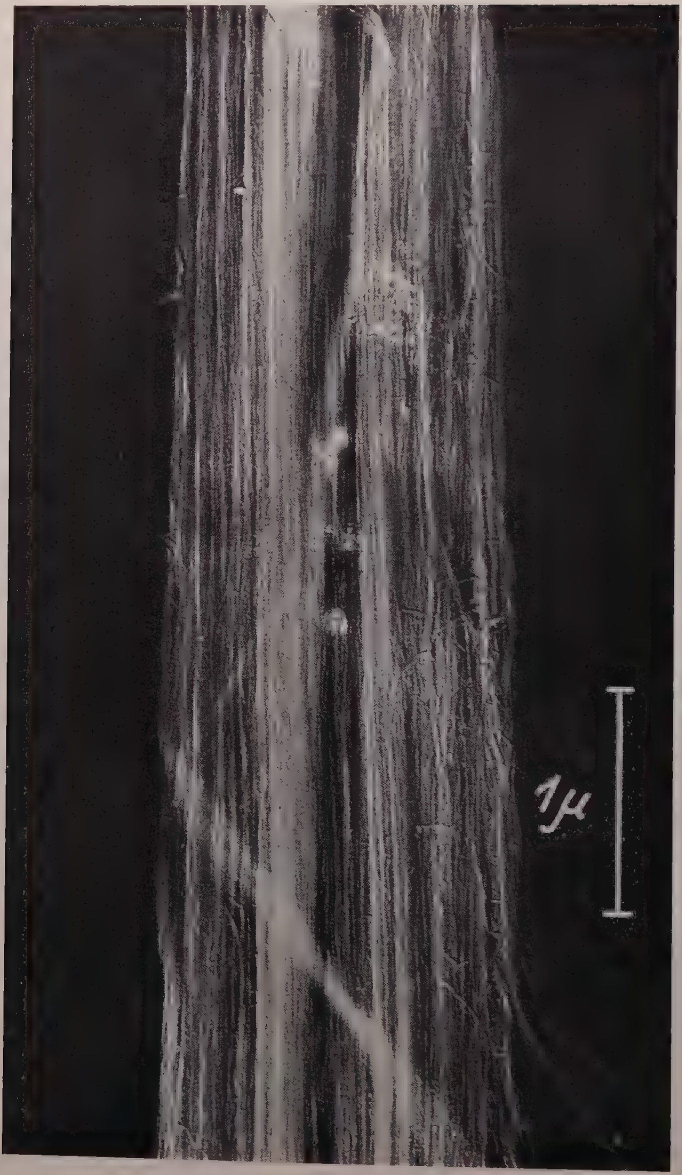

Fig. 4. Viscoseseide (VIV) mit Ultraschall behandelt.

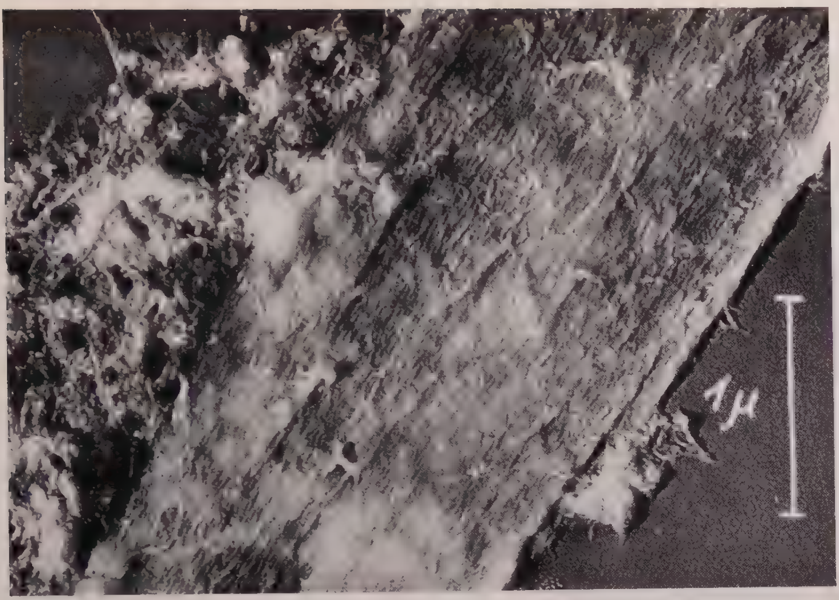

Fig. 5. Isotrope Viscosefiber (VVI) mit Ultraschall behandelt.

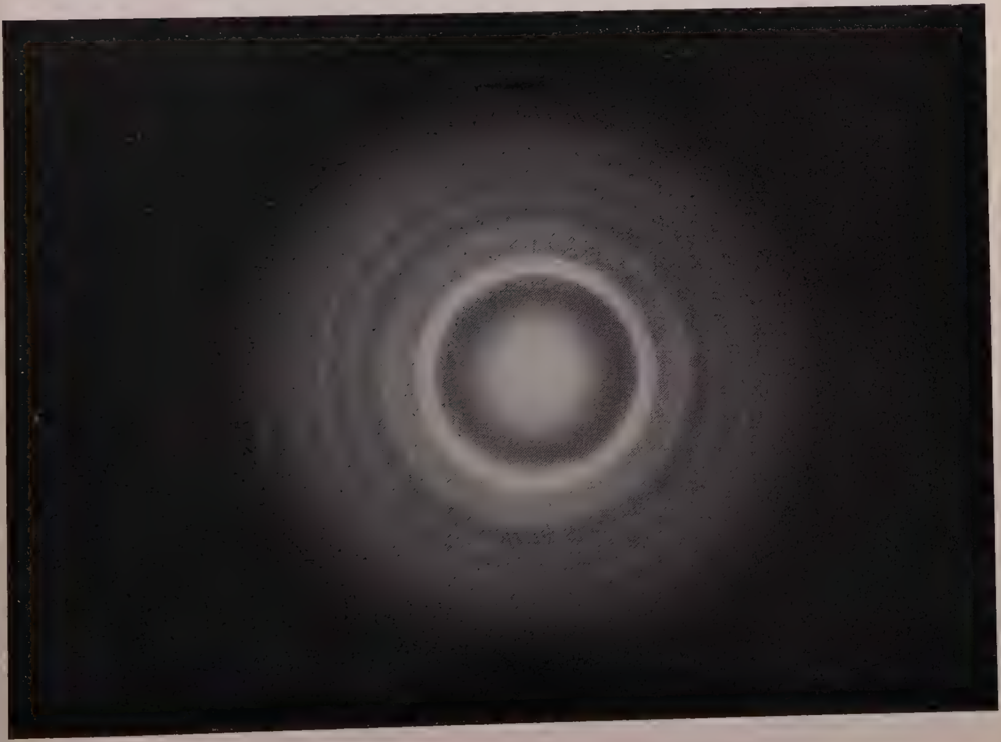

Fig. 6. Elektronenbeugung von hydrolysierter Viscoseseide. (2,5 × Lichtoptisch nachvergrössert.)

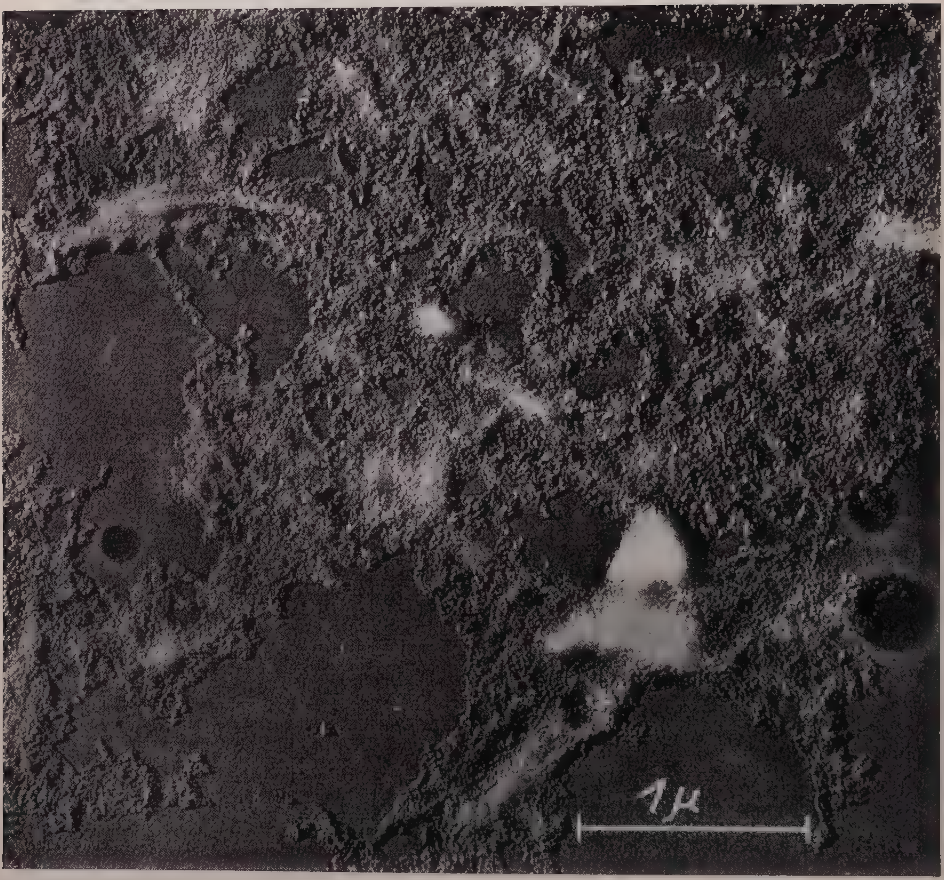

Fig. 7. Hydrolysierte Viscoseseide (V_{III} 1 Std. mit 2,5 n H_2SO_4).

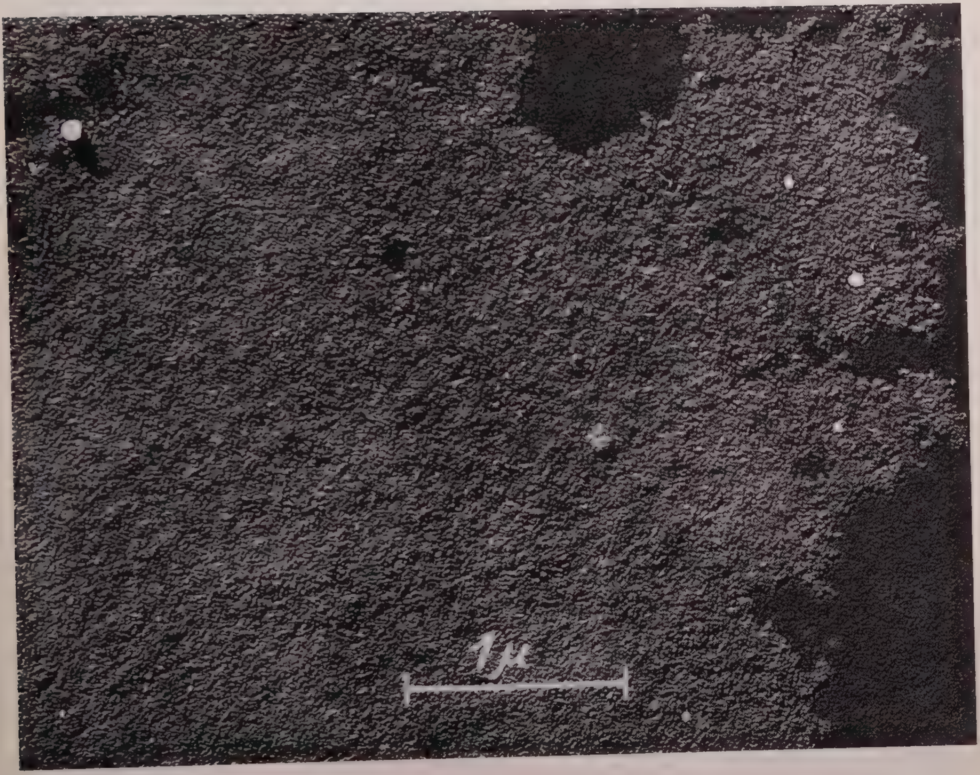

Fig. 8. Hydrolysierte isotrope Viscosefiber (VVI 3 Std. mit 2,5 n H_2SO_4).

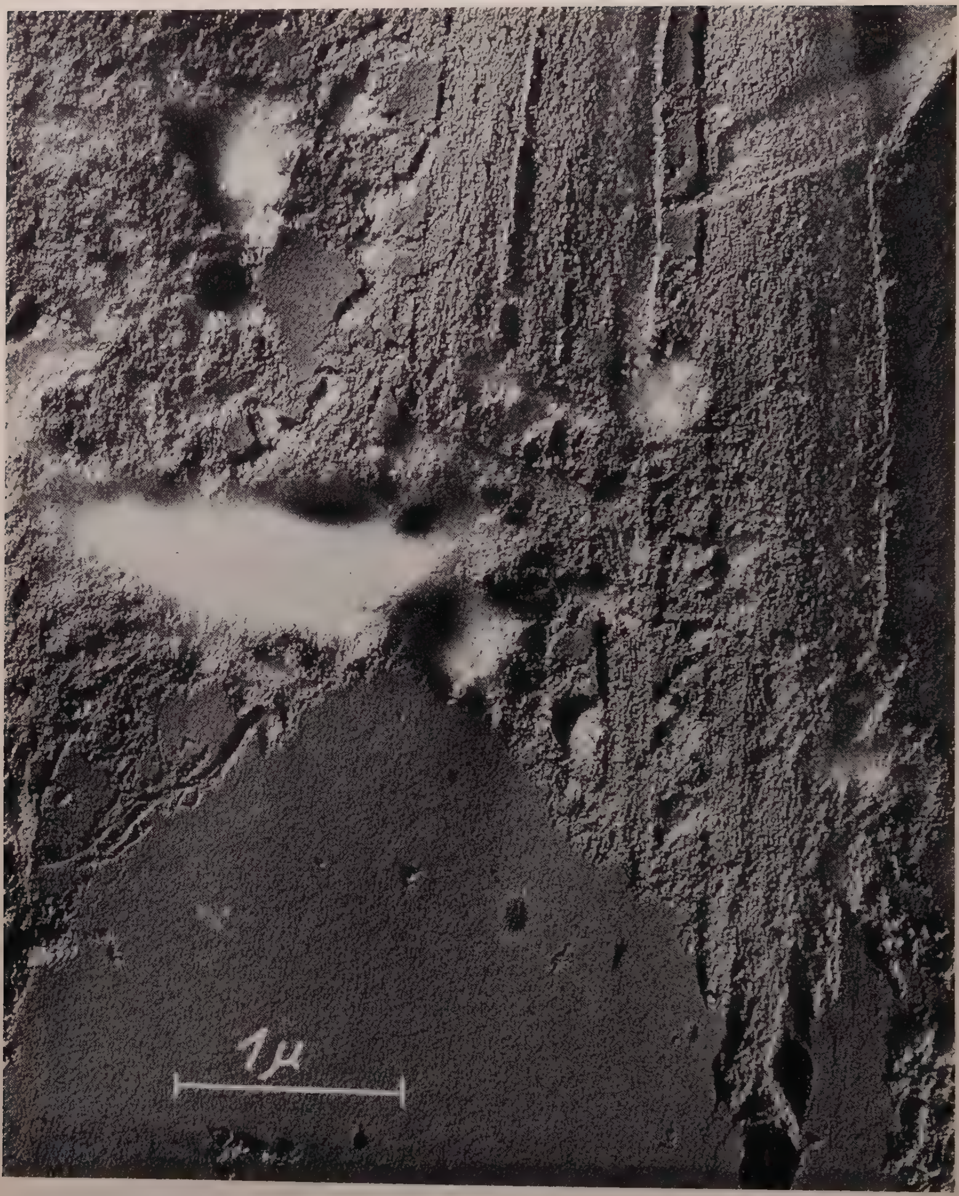

Fig. 9. Anhydrolysierte Viscoseseide mit Ultraschall behandelt (V_{II} 2 Min. mit 2,5 n H_2SO_4).

ARKIV FÖR KEMI Band 2 nr 41

Vorgelegt am 7. Juni 1950

Über Styrylthioglykolsäuren

Von Bror Holmberg

Übersicht

Aus ligninchemischen Gründen im Bedürfnis einiger Kenntnis über die Eigenschaften einer β-Arylvinyl-thioglykolsäure versuchten wir vor 15 Jahren im damaligen Laboratorium des Verf.:s[1] solche Säuren durch Umsatz des Phenylacetylens mit Thioglykolsäure darzustellen. Wie bei der Addition des p-Thiokresols an denselben Kohlenwasserstoff gemäss Kohler und Potter (1) entstanden dabei zwei isomere Verbindungen in Mengenverhältnissen, die merkbar von der Reaktionstemperatur abhängig waren, und welche bei höherer Temperatur ineinander übergehen konnten. Beide Formen schmelzen bei 89°—90° und zeigen das gleiche Aussehen und ungefähr die gleichen Löslichkeiten, während die Löslichkeiten ihrer Bariumsalze hinreichend verschieden sind, um eine bequeme Trennung zu erlauben. Bei weiterer Einwirkung von Thioglykolsäure gehen beide, obgleich nur sehr langsam, in Phenylacetaldehyd-mercaptalessigsäure über, woraus hervorgeht, dass die Addition jener Säure an das Phenylacetylen ähnlich wie bei ihrer Umsetzung mit dem Styrol (2) unter Anlagerung des Schwefels in β-Stellung zum Phenylkern erfolgt. Die erhaltenen Produkte sind also als geometrisch isomere β-Phenylvinyl- oder Styryl-thioglykolsäuren anzusehen, aber da es nicht möglich ist, sichere Konfigurationsformeln für sie aufzustellen, wird die in überwiegender Menge entstehende und das weniger schwerlösliche Bariumsalz gebende provisorisch als A- und die andere als B-Styrylthioglykolsäure bezeichnet.

Auf Grund der glatten Bildung des β-Phenylthioacetats aus Styrol und Thioessigsäure (3) wurde auch versucht[2], in analoger Weise Styrylthioacetate darzustellen, um aus diesen durch Verseifungen und Umsetzungen der dabei zu erwartenden Mercaptide mit Monohalogenacetaten zu Styrylthioglykolsäuren zu kommen. Aus Phenylacetylen und Thioessigsäure erhielten wir hierbei, ähnlich wie kürzlich Behringer (4), ein flüssiges Produkt, welches bei höherer Temperatur[3] teilweise in ein kristallisiertes vom Schmp. 44°—46° überging, während Heilbron und Mitarbeiter (5) unter den von ihnen gewählten Versuchsbedingungen ein festes, bei 32° schmelzendes Produkt erhielten. Bei der Verseifung usw. lieferten indessen beide Formen B-Styrylthioglykolsäure, was auf einer Isomerisierung von durch Hydrolyse freigemachtem Mercaptan gemäss dem Schema:

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[1] Anlässlich der Diplomarbeit des Hrn. Civilingenieurs C. H. Jacobson 1935—36.

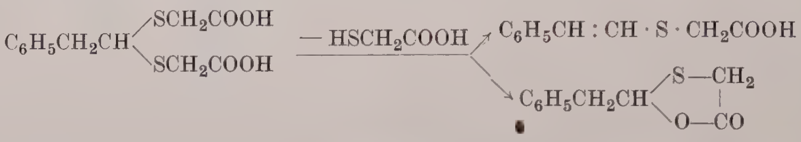
[2] Die ersten Versuche hierüber wurden im hiesigen Institut im Jahre 1946 von Hrn. Mag. Scient. J. Wangel ausgeführt, wonach die Untersuchung unter Mithilfe von Hrn. Lic. Phil. B. Weibull und Frau Majbritt Vestergaard fortgesetzt wurde.

[3] Gemäss Behringer (a.a.O.) kann die flüssige Form schon bei gewöhnlicher Temperatur in die feste übergehen.

$$\text{A}-C_6H_5CH:CH\cdot SH \rightleftharpoons C_6H_5CH_2CHS \rightleftharpoons \text{B}-C_6H_5CH:CH\cdot SH$$

beruhen dürfte.[1]

Bei einem Versuch, Styrylthioglykolsäuren durch thermische Spaltung der Phenylacetaldehyd-mercaptalessigsäure darzustellen, wurden zwar diese Säuren erhalten, aber die Ausbeute war nur gering und als Hauptprodukt entstand das 2-Benzyl-1,3-oxthiophanon-5 oder das innere Anhydrid der Phenylacetaldehydhalbmercaptalessigsäure:



Ein ähnlicher Versuch mit der Acetophenon-mercaptolessigsäure lieferte keine ungesättigte Säure, sondern nur das 2-Methyl-2-phenyl-1,3-oxthiophanon-5.

Bestimmungen der Affinitätskonstanten[2] der Styrylthioglykolsäuren ergaben keine Anhaltspunkte zur Beurteilung ihrer Konfiguration, denn für beide Formen wurde praktisch derselbe Wert, nämlich $k = 3{,}1 \times 10^{-4}$ für die A-Säure und $k = 3{,}2 \times 10^{-4}$ für die B-säure, erhalten. — Von den dargestellten Salzen der Säuren ist das Natriumsalz der A-Säure in Wasser sehr leicht löslich und enthält 1 H_2O, während das entsprechende Salz der B-Säure ziemlich schwerlöslich ist und sich ohne Kristallwasser abscheidet. Das Bariumsalz der A-Säure ist ziemlich schwerlöslich und enthält 3 H_2O, wogegen das gleiche Salz der B-Säure nur sehr wenig löslich und wasserfrei ist.

Bromierungen der Styrylthioglykolsäuren ergaben Dibromide, von denen nur ein B-Derivat in einigermassen reinem Zustande gewonnen wurde, denn sie werden schnell schon durch die Feuchtigkeit der Luft zersetzt; als dabei entstehende Produkte konnten nur kleine Mengen von nicht ganz einheitlichen Bromstyrylthioglykolsäuren nachgewiesen werden. — Oxydationen mit Wasserstoffsuperoxyd und mit Kaliumpersulfat ergaben bei der A-Styrylthioglykolsäure ein viskoses Öl, während hierbei aus der B-Säure eine kristallisierte Styrylsulfinoessigsäure vom Schmp. 109°–110° gewonnen werden konnte. Beim Destillieren eines Gemisches dieser Säure und verdünnter Schwefelsäure mit Wasserdampf entstand ein schwach öliges Destillat, welches bei der Klärung mit Äther und Behandlung der entstandenen, ätherischen Lösung mit Jod eine geringe Menge eines Distyryldisulfids vom Schmp. 40°–42° lieferte, während aus dem Rückstand im Destillierkolben eine Säure vom Schmp. 113°–115° und der Zusammensetzung einer bis-Styrylsulfidoessigsäure, $(C_6H_5CH:CH\cdot S)_2:CHCOOH$, isoliert werden konnte.

Nach kurzem Erhitzen einer wässerigen Lösung des Natriumsalzes der A-Styrylthioglykolsäure mit monobromessigsaurem Natron und Ansäuern der erkalteten Lösung wurde eine Styrylthetinessigsäure erhalten, welche als Rohprodukt bei 128°–129°, nach der Umkristallisation aus Wasser aber bei 119°–120° (in beiden Fällen unter heftiger Gasentwicklung) schmolz; in der gleichen Weise lieferte die B-Styrylthioglykolsäure eine Thetinsäure vom Schmp. 109°–110°, bzw. 100°–101° (wie oben). Beim Erhitzen dieser Thetinsäuren in neutralen, eigenen oder schwefelsauren Lösungen sind sie verhältnismässig beständig, während sie beim Erhitzen mit Salzsäure in Styrylthioglykolsäure und Monochloressigsäure zerfallen. Wenn ein solches Gemisch mit Wasserdampf destilliert wird, erhält man dagegen im Destillat Phenylacetaldehyd und im Rückstand Thiodiglykolsäure; diese Säure entsteht auch beim Erhitzen der Thetinsäuren in alkalischen Lösungen, aber als zweites Produkt bekommt man hier Phenylglykol.

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[1] Hierauf hofft der Verf. zurückkommen zu können.

[2] Diese Bestimmungen verdankt der Verf. Hrn. Dr. Axel Johansson.

Beschreibung der Versuche

1. Phenylacetylen und Thioglykolsäure

Aus 12,8 g Phenylacetylen und 11 g Thioglykolsäure wurde ein homogenes Gemisch erhalten, welches unter Wärmeentwicklung in ein viskoses Öl überging. Nach dem Erkalten kristallisierte das Öl allmählich und schied dabei 3,5 g A-Styrylthioglykolsäure aus, welche beim Umkristallisieren aus wenig Benzol als naphthalinähnliche Schuppen vom Schmp. 89°–90° und Äquiv.-Gew. 194,5, ber. 194,2, erhalten wurde. Die ölige Mutterlauge des Rohprodukts ergab mit stark verdünnter Kalilauge eine milchige Lösung, welche nach Klärung mittels Äther mit einer auch stark verdünnten Lösung von Chlorbarium versetzt wurde. Hierbei entstand eine feinkörnige Fällung, welche bei der Behandlung mit Äther und Salzsäure vollständig gelöst wurde, wonach die ätherische Schicht bei der freiwilligen Eindunstung eine B-Styrylthioglykolsäure ergab, welche beim Umkristallisieren wie oben auch als glänzende Schuppen vom Schmp. 89°–90°, im Gemisch mit der A-säure aber 64°–67°, und Äquiv.-Gew. 194,2 erhalten wurde.

Bei einigen unter besser definierten Verhältnissen ausgeführten Versuchen wurden zuerst 10,4 g Phenylacetylen unter Eiskühlung und in kleinen Portionen mit 12 g Thioglykolsäure versetzt, wonach das Gemisch eine Woche im Eisschrank aufbewahrt wurde. Bei der Behandlung mit 200 ml Wasser und 150 ml 1-n. Natronlauge lieferte es dann 4,2 g eines mit Äther aufgenommenen Öls, wonach die klare Lösung mit 40 ml 5-n. Salzsäure eine Emulsion ergab, welche eine stark ölige Kristallmasse ausschied. Aus dieser Masse wurde mit 200 ml Wasser und 55 ml 1-n. Natronlauge eine neutrale Lösung erhalten, welche mit 50 ml 0,2-n. Chlorbarium-Lösung 2,2 g einer feinkörnigen Fällung lieferte. Aus dieser wurden wie oben 1,4 g B-Styrylthioglykolsäure vom Schmp. ca 70°–85° erhalten. Das Filtrat des Bariumsalzes lieferte mit 15 ml 5-n. Salzsäure 6,8 g A-Styrylthioglykolsäure vom Schmp. ca. 70°–85°. — Bei einem neuen Versuch wie oben, aber ohne Kühlung und mit der Thioglykolsäure auf einmal zugesetzt, stieg die Temperatur des Gemisches auf 80°, und bei der am folgenden Tag vorgenommenen Aufarbeitung ergab es 3,3 g ungelöstes Öl, 1,8 g B-Säure vom Schmp. 87°–89° und 8,5 g A-Säure vom Schmp. ca. 65°–82°. — Als bei einem dritten Versuch das wie oben zusammengesetzte Reaktionsgemisch 4 Stunden auf dem Wasserbad erhitzt wurde, lieferte es danach 1 g ungelöstes Öl, 4,8 g B-Säure vom Schmp. 85°–87° und 6,8 g A-Säure vom Schmp. ca. 70°–85°.

Beim Umkristallisieren roher A-Styrylthioglykolsäuren aus Benzol und Petroläther wurden Produkte vom Schmp. 88°–90° erhalten, und als eine Lösung von 22 g davon in 50 ml warmem Benzol mit 40 ml Petroläther versetzt und das Gemisch im Eisschrank gekühlt wurde, schied es 20 g Kristallschuppen vom Schmp. 89°–90° (Präp. A) aus. In derselben Weise lieferten 16 g B-Säure vom

Schmp. 88°–89,5° beim Umkristallisieren aus 50 ml Benzol und 20 ml Petroläther 14,5 g Kristallschuppen vom Schmp. 89°–90° (Präp. B).

Für $C_6H_5CH : CH \cdot S \cdot CH_2COOH = C_{10}H_{10}O_2S$ (194,24):

ber.: C 61,83, H 5,19, S 16,51 %, Äquiv.-Gew. 194,2,
gef. (A): » 61,92, » 5,12, » 16,45 », » » 194,5,
» (B): » 61,83, » 5,13, » 16,48 », » » 194,0.

Nach 3-stündigem Erhitzen von 2,0 g der A-Styrylthioglykolsäure auf dem Wasserbad schmolz das beim Erkalten erstarrte Material bei ca. 65°–83° und zeigte das Äquiv.-Gew. 205,7. Mit 9,5 ml 1-n. Natronlauge lieferten 1,8 g davon eine schwach alkalische Emulsion, welche nach der Klärung mittels Äther mit 200 ml 0,05-n. Chlorbarium-Lösung versetzt wurde, wobei nur 0,05 g eines Pulvers ausgefällt wurden; die daraus wie oben freigemachte Säure schmolz bei ca. 85°–89°, im Gemisch mit der B-Styrylthioglykolsäure bei ca. 86°–89°. — Nach 3 Stunden bei 120°–125° wurden in der gleichen Weise aus 2,0 g der A-Säure 1,1 g Bariumsalz und daraus 0,7 g B-Säure vom Schmp. 85°–89°, im Gemisch mit der A-Säure 62°–67° und mit reiner B-Säure 85°–89°, und mit dem Äquiv.-Gew. 196,1 erhalten. Das Filtrat des Bariumsalzes lieferte beim Ansäuern 0,6 g Kristallschuppen vom Schmp. ca. 70°–80°, im Gemisch mit der A-Säure ca. 70°–85° und mit der B-Säure 62°–68°, und vom Äquiv.-Gew. 195,0. — Aus 2,0 g B-Styrylthioglykolsäure wurden wie oben nach 3 Stunden bei 120°–125° 1,6 g Bariumsalz gewonnen; die daraus regenerierte Säure wog 1,1 g, schmolz bei 88°–90°, auch im Gemisch mit dem Ausgangsmaterial, und zeigte das Äquiv.-Gew. 195,4. Aus dem Filtrat des Bariumsalzes wurden mit Salzsäure 0,3 g unreine A-Styrylthioglykolsäure vom Schmp. ca. 65°–75° und Äquiv.-Gew. 202,5 gewonnen.

Die Styrylthioglykolsäuren reagieren nur sehr träge weiter mit der Thioglykolsäure, denn als 2,1 g der A-Säure in gelinder Wärme mit 2,2 g jener Säure versetzt wurden, entstand ein homogenes Gemisch, welches nach einem Monat bei gewöhnlicher Temperatur unverändert war und dann mit 40 ml 1-n. Natronlauge eine opaleszierende Lösung lieferte. Diese Lösung wurde von Salzsäure emulgiert und schied dann 1,9 g eines mit 2 × 25 ml Wasser gewaschenen Öls aus; bei der Umfällung aus seiner Lösung in Natronlauge und Impfen des dabei wieder ausgeschiedenen Öls mit einer Spur von Phenylacetaldehyd-mercaptalessigsäure wurden 0,3 g Kristallpulver von Schmp. ca. 94°–98°, nach Umkristallisieren aus Benzol 98°–100°, auch im Gemisch mit der genannten Mercaptalsäure, und vom Äquiv.-Gew. 144,2, ber. 143,1, erhalten. — Ganz ähnlich verhielt sich die B-Styrylthioglykolsäure gegenüber der Thioglykolsäure; auch hier wurden 0,3 g Phenylacetaldehyd-mercaptalessigsäure vom Schmp. wie oben und Äquiv.-Gew. 144,4 erhalten.

2. Phenylacetylen und Thioessigsäure

Als im Laufe von 2 Stunden und unter Eiskühlung 20,4 g Phenylacetylen mit 16 g Thioessigsäure vom Alkali-Äquiv.-Gew. 75,6 und J-Äquiv.-Gew. 78,5 versetzt wurden, entstand ein homogenes Gemisch, welches 5 Tage im Eisschrank verwahrt wurde, wonach ihm mit 3 × 50 ml Wasser Säuren, entsprechend 16,5 + 3,0 + 1,5 = 21 ml 1-n. Natronlauge entzogen wurden. Das dabei ungelöst verbliebene Öl wog 34 g und enthielt nach dem Trocknen mit geschmolzenem Chlorcalcium:

Für $C_6H_5CH : CH \cdot S \cdot COCH_3 = C_{10}H_{10}OS$ (178,24):

ber.: C 67,38, H 5,66, S 17,99 %,
gef.: » 67,58, » 5,60, » 17,47 ».

Von dem erhaltenen, rohen Styrylthioacetat wurden 6,6 g mit einer Lösung von 4 g Kaliumhydroxyd in 40 ml gew. Alkohol versetzt, wobei unter schwacher Wärmeentwicklung eine gelbliche, milchige Lösung entstand. Diese schied bis zum folgenden Tag eine unbedeutende Menge einer klebrigen Masse ab; die davon abdekantierte Lösung ergab mit einer Lösung von 5,6 g Monobromessigsäure in 40 ml 1-n. Natronlauge ein homogenes Gemisch, welches allmählich in einen Kristallbrei überging. Nach der Eindunstung des Breies im Vakuum über Schwefelsäure auf 45 g Gewicht lieferte er mit 30 ml 5-n. Salzsäure und 100 ml Äther klare Flüssigkeitsschichten, von denen die ätherische nach Zusatz von 100 ml Wasser gegen Phenolphthalein 48 ml 1-n. Natronlauge verbrauchte und dann bei der freiwilligen Abdunstung des Äthers als Rückstand nur 0,3 g eines klebrigen Öls zurückliess. Die erhaltene, neutrale Wasserlösung ergab beim Ansäuern eine Emulsion, welche 4,6 g B-Styrylthioglykolsäure als Kristallschuppen vom Schmp. 82°–85°, im Gemisch mit der A-Säure 60°–70° und mit der B-Säure 83°–87°, ausschied. Nach der Umkristallisation aus Benzol schmolz die Säure bei 88°–89,5° und enthielt 16,39, ber. 16,51, % S.

Beim Destillieren von 26 g des oben erhaltenen Thioacetats wurden zuerst in 15 Minuten und zum Schluss bei 0,3 mm Druck, 130° im Bad und 97° im Dampf 4 g und dann bei 0,25 mm Druck und 140°, bzw. 95° Temperatur 15 g farblose oder schwach gelbliche Destillate und 6 g eines dunkelbraunen, dickflüssigen Rückstands erhalten. Die Hauptfraktion, welche weder sogleich noch nach 2 Monaten bei gewöhnlicher Temperatur im Eisschrank Kristalle ausschied, zeigte $d_4^{20} = 1{,}1137$, $n_D^{20} = 1{,}6175$, woraus sich die Mol.-Refr. zu 56,03 und die Atom-Refr. des Schwefels zu 11,71 ergibt. Die Analyse lieferte:

ber.: C 67,38, H 5,66, S 17,99 %; **gef.:** C 67,41, H 5,62, S 18,07 %.

Bei einem neuen Versuch wurden 20,4 g Phenylacetylen ohne Kühlung mit 16 g Thioessigsäure in Portionen von je ca. 5 g versetzt, wobei die Temperatur des Reaktionsgemisches nach dem ersten Zusatz auf 66° und nach dem letzten auf 63° stieg. Nach Waschen wie oben, wobei Säuren, entsprechend 18 ml 1-n. Lauge dem öligen Produkt entzogen wurden, wog dieses 33 g, enthielt 16,77, ber. 17,99, % S und ergab bei der Vakuumdestillation eine Hauptfraktion vom $\text{Siedep.}_{0,2} = 94°—95°$, welche 18 g wog, im Eisschrank keine Tendenz zum Kristallisieren zeigte und 17,97, ber. 17,99, % S enthielt. Nach einer Stunde bei 150° ergaben 27 g solcher Fraktionen bei einem Druck von 0,1—0,2 mm, einer Badetemperatur von 120°—135° und einer Temperatur im Dampf von 90°—91° zuerst 12,9 und dann 12,4 g Destillate, welche im Eisschrank 4,4, bzw. 5,9 g Kristallprismen ausschieden. Von den Mutterlaugen davon wurden 12,5 g 2 Stunden wie vorher erhitzt, wonach sie 10,7 g eines Destillats lieferten, welches im Eisschrank 5,9 g Kristalle ausschied. Als 9,4 g der erhaltenen Kristalle aus 50 ml gew. Alkohol umkristallisiert wurden, schied die Lösung beim Erkalten 5,6 g schmale Prismen vom Schmp. 44°—46° aus, welche auch die Zusammensetzung eines Styrylthioacetats zeigten:

ber.: C 67,38, H 5,66, S 17,99 %; gef.: C 67,42, H 5,57, S 18,02 %.

Als 7 g des kristallisierten Thioacetats eine Stunde auf 150° gehalten worden waren, wurden beim Erkalten nur 4,5 g Prismen vom Schmp. wie vorher erhalten, während die Mutterlauge nach der Verseifung und dann beim Umsatz mit monobromessigsaurem Natron wie oben 1,1 g einer B-Styrylthioglykolsäure vom Schmp. 86°—89°, auch im Gemisch mit B-säure, und mit 16,40, ber. 16,51, % S lieferte. — Dieselbe Säure wurde aus dem kristallisierten Thioacetat erhalten, als 4,4 g davon mit 2.8 g Kaliumhydroxyd in 10 ml Wasser und 30 ml Alkohol behandelt wurden und das entstandene Mercaptid dann wie oben mit Monobromacetat umgesetzt wurde, denn die danach erhaltene Fällung mit Salzsäure, welche 4,5 g wog, schmolz für sich bei ca. 78°—86°, im Gemisch mit der A-Säure bei ca. 60°—64° und mit der B-Säure bei ca. 80°—88°. Mit 100 ml Wasser und 21 ml 1-n. Natronlauge lieferte dieses Produkt eine schwach alkalische, etwas milchige Lösung, welche nach der Klärung mittels Äther mit einer Lösung von 2,7 g Chlorbarium in 110 ml Wasser eine pulverige Fällung von 4.8 g Gewicht ergab. Das Filtrat davon lieferte beim Ansäuern 0,25 g Kristallschuppen vom Schmp. 88°—89°,5, im Gemisch mit der A-Säure 63°—66° und mit der B-Säure 88°—89°,5; aus dem Bariumsalz wurden 3,4 g Schuppen vom Schmp. 88°—90°, im Gemisch mit der A-Säure 64°—67° und mit der B-Säure 88°,5—90°, und mit 16,29, ber. 16,51, % S erhalten.

3. Thermische Zersetzungen der Phenyläthyliden-bis-thioglykolsäuren

Als 9,6 g β-Phenyläthyliden-bis-thioglykolsäure (Phenylacetaldehyd-mercaptalessigsäure) in 30 Minuten und unter einem Druck von 0,9—0,6 mm auf 175° erhitzt wurden, destillierten 1,1 g eines farblosen Öls über, und in der gleichen Zeit wurden dann bei 0,6—0,5 mm Druck und 200° noch 6,7 g Destillat und 1,5 g Rückstand erhalten. Der Rückstand bestand aus einer braunen, halbfesten Masse, aus welcher nichts Einheitliches isoliert werden konnte. Die beiden Destillate wurden zusammen in Äther gelöst und die Lösung mit 75 ml 1-n. Sodalösung geschüttelt, wonach die dabei entstandene, wässerige Lösung unter Entwicklung von Kohlendioxyd mit Salzsäure eine Emulsion lieferte, welche 0,7 g eines Gemisches von Styrylthioglykolsäuren von Schmp. ca. 62°—85°, im Gemisch mit der A-Säure ca. 62°—66° und mit der B-Säure ca. 65°—86°, ausschied.

Für $C_6H_5CH:CH \cdot S \cdot CH_2COOH = C_{10}H_{10}O_2S$ (194,24):

ber.: C 61,83, H 5,19, S 16,51 %, Äquiv.-Gew. 194,2,
gef.: » 61,84, » 5,17, » 16,45 », » » 194,4.

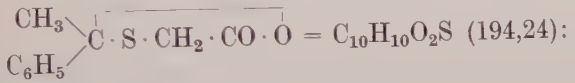
Eine Probe der Mutterlauge dieses Produkts verbrauchte Jod in einer Menge, welche einem totalen Gehalt an Thioglykolsäure von 2,6 g entsprach.

Die mit Sodalösung gewaschene Ätherlösung wurde mit geschmolzenem Chlorcalcium getrocknet und lieferte dann beim Abdestillieren des Äthers 4,1 g eines ölförmigen Extrakts, welcher bei 0,5 mm Druck und 110°—115° 3,2 g Destillat und 0,8 g Rückstand in der Form eines viskosen Öls ergab. Beim Umdestillieren des Destillats wurden 2,8 g 2-Benzyl-1,3-oxthiophanon-5 vom Siedep.$_{0,45}$ = 110°—112° und d_4^{20} = 1,2331 erhalten.

Für $C_6H_5CH_2\overline{CH \cdot S \cdot CH_2 \cdot CO \cdot O} = C_{10}H_{10}O_2S$ (194,24):

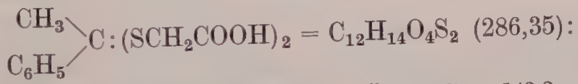
ber.: C 61,83, H 5,19, S 16,51 %,
gef.: » 61,83, » 5,05, » 16,47 » .

Nach langsamem Erhitzen von 10 g α-Phenyläthyliden-bis-thioglykolsäure (Acetophenon-mercaptolessigsäure) auf 160° wurden bei dieser Temperatur und einem Druck von ca. 0,6 mm 8,8 g eines ölförmigen Destillats und 1,1 g eines gelbbraunen, klebrigen Rückstands erhalten. Aus diesem Rückstand konnte nur ein wenig unverändertes Ausgangsmaterial isoliert werden; bei der Behandlung des Destillats mit 42 ml 1-n. Natronlauge entstand eine schwach alkalische Wasserlösung, welche beim Ansäuern nur opaleszierend wurde, während 5 g Öl ungelöst verblieben. Dieses Öl ergab beim Destillieren 4,5 g 2-Methyl-2-phenyl-1,3-oxthiophanon-5 vom Siedep.$_{0,5}$ = 96°—99°.

Für 
$\genfrac{}{}{0pt}{}{CH_3}{C_6H_5}\!>\!\overline{C \cdot S \cdot CH_2 \cdot CO \cdot O} = C_{10}H_{10}O_2S$ (194,24):

ber.: C 61,83, H 5,19, S 16,51 %,
gef.: » 61,97, » 5,26, » 16,34 » .

Beim Aufbewahren begann dieses Oxthiophanon allmählich Kristalle auszuscheiden, welche nach 5 Monaten abgesaugt und mit Benzol gewaschen wurden, wonach sie 1,1 g wogen und als zurückgebildete Acetophenon-mercaptolessigsäure vom Schmp. 134°—135° identifiziert wurden.

Für 
$\genfrac{}{}{0pt}{}{CH_3}{C_6H_5}\!>\!C:(SCH_2COOH)_2 = C_{12}H_{14}O_4S_2$ (286,35):

ber.: C 50,33, H 4,93, S 22,39 %, Äquiv.-Gew. 143,2,
gef.: » 50,59, » 4,91, » 22,32 », » » 143,7.

4. Affinitätskonstanten und Salze der Styrylthioglykolsäuren

Zwecks Bestimmung der Affinitätskonstanten der beiden Styrylthioglykolsäuren wurden je 2,000 Millimole dieser Säuren gegen Phenolphthalein mit Natronlauge neutralisiert, und nach Verdünnungen mit Wasser und Zusetzen von x Millimolen Chlorwasserstoff (als 1/10-n. Lösung) wurden mittels einer Glaselektrode die pH-Werte der Gemische bestimmt. Die Gesamtvolumina der Lösungen betrugen zum Schluss je 400 ml, und die aus den Messungen erhaltenen Werte der Affinitätskonstanten, $k = [\overset{+}{H}] \times [\text{Salz}]/[\text{Säure}]$, sind aus der Tabelle auf der folgenden Seite ersichtlich.

Im Mittel erhält man für die A-Säure $k = 3{,}1 \times 10^{-4}$ und für die B-Säure $k = 3{,}2 \times 10^{-4}$.

Bei der freiwilligen Eindunstung einer Lösung von 2,0 g der A-Styrylthioglykolsäure in 10,3 ml 1-n. Natronlauge wurde ein hauptsächlich aus Effloreszenzen bestehender Rückstand erhalten, welcher beim Umkristallisieren aus gew. Alkohol haarfeine Nädelchen ergab, die in lufttrockener Form analysiert wurden.

x		p_H		$k \times 10^4$	
A	B	A	B	A	B
0,136	0,158	4,65	4,60	3,1	3,0
0,242	0,223	4,35	4,40	3,2	3,2
0,352	0,355	4,17	4,16	3,2	3,2
0,499	0,486	3,99	3,97	3,1	3,3
0,604	0,618	3,87	3,83	3,1	3,3
0,715	0,749	3,77	3,70	3,0	3,3
0,827	0,881	3,68	3,60	3,0	3,2
0,939	1,012	3,59	3,50	2,9	3,1
1,051	1,080	3,50	3,44	2,9	3,1

Für $C_6H_5CH:CH \cdot S \cdot CH_2COONa, H_2O = C_{10}H_{11}O_3SNa$ (234,24):

ber.: S 13,69, Na 9,82, H_2O 7,69 %,
gef.: » 13,88, » 9,82, » 7,48 ».[1]

Als eine Lösung von 2,0 g derselben Säure in 10 ml Wasser und 10,2 ml 1-n. Natronlauge mit 80 ml 1/8-n. Chlorbarium-Lösung versetzt wurde, entstand ein opaleszierendes Gemisch, welches bis zum folgenden Tag in einen Brei von kleinen Kristallschuppen überging. Nach Absaugen, Waschen mit Wasser und Trocknen an der Luft wogen die Schuppen 1,6 g und ergaben bei der Analyse:

Für $(C_6H_5CH:CH \cdot S \cdot CH_2COO)_2Ba, 3\,H_2O = C_{20}H_{24}O_7S_2Ba$ (577,88):

ber.: Ba 23,77, H_2O 9,35 %; gef.: Ba 23,65, H_2O 9,36 %.

Von 2,0 g der B-Styrylthioglykolsäure, 10 ml Wasser und 10,2 ml 1-n. Natronlauge wurde beim Erwärmen eine Lösung erhalten, welche beim Erkalten in einen Brei von haarfeinen Nädelchen überging. Nach Absaugen, Waschen mit 10 ml Wasser und Trocknen an der Luft wogen diese 1,35 g und verloren im Vakuum über Phosphorsäureanhydrid 0,61 % an Gewicht, wonach sie analysiert wurden.

Für $C_6H_5CH:CH \cdot S \cdot CH_2COONa = C_{10}H_9O_2SNa$ (216,23):

ber.: S 14,83, Na 10,64 %; gef.: S 14,57, Na 10,40 %.

Das Filtrat des Natriumsalzes wurde mit 40 ml 0,2-n. Chlorbarium-Lösung versetzt, wobei 1,0 g eines mit 20 ml Wasser gewaschenen Kristallpulvers ausgeschieden wurde, welches in lufttrockener Form analysiert wurde.

Für $(C_6H_5CH:CH \cdot S \cdot CH_2COO)_2Ba = C_{20}H_{18}O_4S_2Ba$ (523,83):

ber.: Ba 26,22 %; gef.: Ba 26,32 %.

5. Oxydationsprodukte der Styrylthioglykolsäuren

A. Bromierungsprodukte

Als eine Lösung von 2,0 g der A-Styrylthioglykolsäure in 10 ml Eisessig mit 10 ml einer 2-n. Lösung von Brom in demselben Lösungsmittel versetzt wurde, trat sogleich und unter schwacher Wärmeentwicklung Entfärbung ein, wonach noch ein ml derselben Lösung nur sehr langsam verbraucht wurde. Bei Zusatz von 100 ml Wasser ergab das Reaktionsgemisch eine Emulsion, welche bis zum folgenden Tag eine teigige Kristallmasse ausschied. Nach dem Trocknen auf Tonscherben wog das Produkt 1,6 g und lieferte bei der Umkristallisation aus 10 ml Benzol 0,5 g Kristallschuppen vom Schmp. 112°–114° und der annähernden Zusammensetzung einer Bromstyrylthioglykolsäure.

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[1] Wie beim folgenden Salz Gewichtsverlust im Vakuum über Phosphorsäureanhydrid.

Für $C_{10}H_9O_2BrS$ (273,15):

ber.: C 43,97, H 3,32, Br 29,26, S 11,74 %,
gef.: » 44,39, » 3,39, » 28,42, » 11,71 ».

Eine Lösung von 0,2973 g des analysierten Präparats in 10 ml Alkohol und 10 ml Wasser verbrauchte gegen Phenolphthalein 11,00 ml 0,1000-n. Natronlauge, was dem Äquiv.-Gew. 270,3, ber. 273,15, entspricht. Nach dem Zusatz von weiteren 11,37 ml der Lauge verbrauchte die Lösung am folgenden Tag 4,87 ml 0,1000-n. Salzsäure und von noch 10,04 ml der Lauge wurden bis zum nächsten Tag 4,04 ml davon verbraucht, woraus sich schliesslich ein Äquiv.-Gew. von 138 ergibt.

Aus 2,0 g B-Styrylthioglykolsäure wurde wie beim vorigen Versuch mit Brom eine farblose Lösung erhalten, welche bei der freiwilligen Eindunstung in einem Luftstrom einen teilweise kristallisierten Rückstand lieferte. Nach Trocknen wie oben wog dieser 2 g, und nach zweimaligem Umkristallisieren durch Fällung mit Petroläther aus seiner Lösung in Benzol bestand das Produkt aus 0,6 g farblose Prismen vom Schmp. 109°–112° von derselben Zusammensetzung wie oben.

Für $C_{10}H_9O_2BrS$ (273,15):

ber.: C 43,97, H 3,32, Br 29,26, S 11,74 %,
gef.: » 44,45, » 3,29, » 27,22, » 11,87 ».

Von 2,0 g der B-Säure in 20 ml Benzol und 1,65 g tropfenweise zugesetztem Brom wurde eine farblose Lösung erhalten, welche bis zum folgenden Tag in einen Brei von farblosen, glänzenden Blättchen überging. Nach Absaugen, Waschen mit 5 ml Benzol, Abpressen zwischen Filtrierpapier und Trocknen im Vakuum über Phosphorsäureanhydrid schmolz das Produkt bei 101°–103° unter Aufschäumen und enthielt:

Für $C_6H_5CHBrCHBr \cdot S \cdot CH_2COOH = C_{10}H_{10}O_2Br_2S$ (354,07):

ber.: Br 45,14, S 9,05 %; gef.: Br 44,33, S 9,00 %.

Eine Lösung von 0,4066 g des analysierten Präparats in 10 ml Alkohol ergab mit Phenolphthalein einen unscharfen Umschlag für 31,00 ml 0,1000-n. Natronlauge, und nach Zusatz von noch 10,00 ml der Lauge wurde sie am folgenden Tag durch 7,20 ml 0,1000-n. Salzsäure neutralisiert, woraus sich das Äquiv.-Gew. 120 ergibt. Beim Titrieren mit einer 0,0985-n. J-Lösung wurden dann mit unscharfem und gleitendem Umschlag 11,2 ml davon verbraucht, entsprechend dem Äquiv.-Gew. 369.

B. Perhydrol- und Persulfatprodukte

Beim Oxydieren der A-Styrylthioglykolsäure mit Perhydrol oder mit Persulfat wie unten konnten praktisch keine kristallisierten Produkte gewonnen werden.

Als eine Lösung von 2,0 g B-Styrylthioglykolsäure in 10 ml Aceton mit 2 g

Perhydrol versetzt wurde, entstand ein homogenes Gemisch, welches am folgenden Tag unverändert war und danach bei freiwilliger Eindunstung ein viskoses Öl zurückliess. Dieses Öl ging allmählich in eine zum Teil kristallisierte Masse über, welche auf Tonscherben 1,3 g Kristalle vom Schmp. 96°–100° lieferte. Beim Fällen seiner Lösung in abs. Alkohol mit Benzol ergab das Produkt im Eisschrank Nadeln von Schmp. 109°–110° (Schmelze blasig), welche die Zusammensetzung einer Styrylsulfinoessigsäure zeigten.

Für $C_6H_5CH:CH \cdot SO \cdot CH_2COOH = C_{10}H_{10}O_3S$ (210,24):

ber.: C 57,13, H 4,79, S 15,25 %, Äquiv.-Gew. 210,2,
gef.: » 57,03, » 4,79, » 15,26 », » » 210,1.

Nach Zusatz von 6 g Kaliumpersulfat zu einem Gemisch von 4,0 g der B-Säure und 9 g Soda in 50 ml Wasser entstand beim Umrühren und unter Kohlendioxyd-Entwicklung und schwachem Erwärmen ein opaleszierendes Gemisch, welches mit 20 ml 5-n. Salzsäure eine Emulsion lieferte, aus der 4 g Sulfinosäure als eine von Öl erstarrte Kristallmasse vom Schmp. 106°–108° wie oben und Äquiv.-Gew. 214,2, ber. 210,2, ausgeschieden wurden. Nach der Umfällung aus ihrer Lösung in Natronlauge schmolz die Säure bei 109°–111° wie oben und ergab dann bei der Analyse:

ber.: C 57,13, H 4,79, S 15,25 %, Äquiv.-Gew. 210,2,
gef.: » 57,21, » 4,76, » 15,21 », » » 209,5.

Von den erhaltenen Sulfinosäuren wurden 4,2 g mit 50 ml 2-n. Schwefelsäure versetzt und das Gemisch unter Beibehaltung des Volumens mit Wasserdampf destilliert, wobei ein anfangs milchiges Destillat erhalten wurde. Dieses wurde mit Äther extrahiert und die ätherische Lösung mit Jod im Überschuss versetzt; nach Entfärbung mit Natriumbisulfit wurden bei der freiwilligen Abdunstung des Äthers 0,7 g eines viskosen Öls erhalten, welches teilweise von 10 ml gew. Alkohol gelöst wurde, wonach das Gemisch im Eisschrank 0,2 g farblose, nadelförmige Prismen ausschied. Diese Prismen schmolzen bei 40°–42° und zeigten die Zusammensetzung eines Distyryldisulfids:

Für $(C_6H_5CH:CH \cdot S)_2 = C_{16}H_{14}S_2$ (270,39):

ber.: C 71,07, H 5,22, S 23,71 %,
gef.: » 70,89, » 5,29, » 23,61 ».

Ein im Destillierkolben zurückgebliebenes, viskoses Öl wurde mit 50 ml Äther aufgenommen und die ätherische Lösung mit 20 ml 0,5-n. Natronlauge geschüttelt, wobei eine wässerige Lösung entstand, welche bei freiwilliger Eindunstung schnell in einen Brei von farblosen Nädelchen überging. Nach Trocknen an der Luft verloren diese im Vakuum über Phosphorsäureanhydrid 4,44. für 1 H_2O ber. 4,89, % an Gewicht und zeigten dann die Zusammensetzung des Natriumsalzes einer bis-Styrylsulfidoessigsäure, bzw. eines Glyoxylsäure-styrylmercaptals:

Für $C_{18}H_{15}O_2S_2Na$ (350,42):

ber.: S 18,30, Na 6,56 %; gef.: S 18,13, Na 6,45 %.

Die freie Mercaptalsäure wurde erhalten, als 0,8 g des Salzes in 20 ml warmem Wasser mit Salzsäure versetzt wurden. Hierbei entstand eine Emulsion, welche

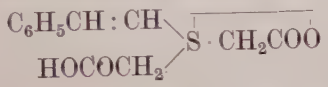
0,7 g einer von Öl erstarrten Kristallmasse vom Schmp. 113°–115° und der erwarteten Zusammensetzung ausschied.

Für $(C_6H_5CH:CH\cdot S)_2:CHCOOH = C_{18}H_{16}O_2S_2$ (328,43):

ber.: C 65,83, H 4,91, S 19,52 %, Äquiv.-Gew. 328,4,
gef.: » 65,98, » 4,90, » 19,44 » , » » 329,6.

6. Styrylthetinessigsäuren

Beim Erhitzen eines Gemisches aus 4,0 g A-Styrylthioglykolsäure in 21 ml 1-n. Natronlauge und 7 g Monobromessigsäure in 50 ml derselben Lauge 1/2 Stunde lang auf dem Wasserbad entstand eine stark opaleszierende Lösung, welche klar und neutral für 17 ml der Lauge wurde. Nach wiederholtem Erhitzen wie vorher wurden noch 7 ml der Lauge verbraucht, und nach dem Erkalten und Zusatz von 10 ml 5-n. Salzsäure schied dann die Lösung 4,5 g eines Pulvers aus, welches mit 25 ml gew. Alkohol behandelt wurde, wonach es 3,5 g wog, bei 128°–129° unter heftigem Aufschäumen schmolz und das Äquiv.-Gew. 249,0, ber. 252,3, zeigte. Beim Umkristallisieren aus 60 ml Wasser lieferten 1,0 g dieses Produkts 0,6 g kleine, glänzende Prismen vom Schmp. 119°–120° wie oben und der Zusammensetzung einer Styrylthetinessigsäure.

Für  $= C_{12}H_{12}O_4S$ (252,28):

$$\begin{matrix} C_6H_5CH:CH \\ HOCOCH_2 \end{matrix} > \overline{S\cdot CH_2COO}$$

ber.: C 57,13, H 4,79, S 12,71 %, Äquiv.-Gew. 252,3,
gef.: » 57,06, » 4,77, » 12,73 » , » » 252,6.

Das alkoholische Filtrat des ungelösten Rohprodukts ergab bei freiwilliger Eindunstung eine teigige Masse, welche von 25 ml Äther fast vollständig gelöst wurde; als die ätherische Lösung mit 20 ml 0,5-n. Natronlauge ausgeschüttelt und die dabei erhaltene Wasserlösung angesäuert wurde, schied dieselbe 0,8 g A-Styrylthioglykolsäure als kleine Kristallschuppen vom Schmp. 86°–89°, im Gemisch mit der A-Säure 87°–89°, und vom Äquiv.-Gew. 194,6, ber. 194,2, aus.

Als ein Gemisch aus 2,5 g mit Alkohol gereinigtem Thetin und 50 ml 2-n. Salzsäure unter Beibehaltung des Volumens mit Wasserdampf destilliert wurde, entstanden in einer Stunde 140 ml eines schwach öligen Destillats, welches beim Extrahieren mit Äther 0,5 g eines öligen Extrakts lieferte. Das Öl wurde in 10 ml Alkohol gelöst und eine Lösung von 1 g chlorwasserstoffsaurem Semicarbazid und 1,3 g Soda in 5 ml Wasser zugesetzt, wobei ein homogenes Gemisch entstand, welches bei der freiwilligen Eindunstung 0,6 g Kristallpulver vom Schmp. 144°–146° ausschied. Nach Umkristallisation aus Benzol schmolz das so erhaltene Phenylacetaldehyd-semicarbazon bei 149°–151°, im Gemisch mit aus authentischem Phenylacetaldehyd in derselben Weise dargestelltem und umkristallisiertem Semicarbazon[1] bei 150°–152°, und enthielt 23,71, ber. 23,72, % N. — Das im Destillierkolben zurückgebliebene Gemisch aus Lösung und einer kleinen Menge eines viskosen Öls wurde dreimal mit 100 ml Äther ausgeschüttelt, wonach dieser 19 + 4 + 1 = 24 ml 1-n. Natronlauge verbrauchte. Die entstandene Wasserlösung schied beim Ansäuern nur wenig klebriges Öl aus, und das Filtrat davon ergab beim Ausäthern eine unreine Thiodiglykolsäure vom Schmp. ca. 120° und Äquiv.-Gew. 81, ber. 75,1 (vergl. unten). — Als dagegen 1,5 g A-Styrylthetinessigsäure im Gemisch mit 50 ml 2-n. Schwefelsäure wie oben destilliert wurden, entstand ein klares Destillat, und die zurückgebliebene, klare Lösung schied nach dem Erkalten und Impfen mit einer bei der freiwilligen Eindunstung kristallisierten Probe davon 1,15 g unveränderte Thetinsäure vom Schmp. 118°–119,5° (unter heftiger Gasentwicklung) und Äquiv.-Gew. 251,0, ber. 252,3, aus.

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[1] Dieses Präparat schmolz bei 153°–154°, während Henle (6) sowie Heilbron und Mitarbeiter (5) für das Phenylacetaldehyd-semicarbazon den Schmp. 156° angeben.

Beim Erhitzen auf dem Wasserbad von 2,5 g A-Styrylthetinessigsäure mit 25 ml 1-n. Salzsäure entstand eine klare Lösung, welche sich schnell emulgierte und nach 2 Stunden eine reichliche Menge eines farblosen Öls ausgeschieden hatte. Nach dem Erkalten wurde das Gemisch ausgeäthert, wobei ihm Säuren, entsprechend 21 ml 1-n. Natronlauge entzogen wurden. Die hierbei entstandene, wässerige Lösung schied beim Ansäuern ein viskoses Öl ab, welches im Eisschrank grösstenteils kristallisierte und dann auf Tonscherben 1,1 g Styrylthioglykolsäure vom Schmp. ca. 80°–85°, im Gemisch mit der A-Säure 82°–87°, und Äquiv.-Gew. 192,6, ber. 194,2, zurückliess. Das Filtrat davon lieferte beim Extrahieren mit 3 × 50 ml Äther, freiwilliger Abdunstung des Äthers und Trocknen des Rückstands im Vakuum über Schwefelsäure 0,95 g Monochloressigsäure, welche das Äquiv.-Gew. 97,5, ber. 94,5, zeigte und nach Erhitzen mit Natronlauge eine kräftige Chlorid-Reaktion ergab. — Nach dem Erhitzen wie oben von 1,3 g Thetinsäure mit 50 ml Wasser war immer noch etwas ungelöstes Material vorhanden; nach dem Erkalten wurden 1,0 g unzersetzte Säure vom Schmp. 119°–120° wie oben und Äquiv.-Gew. 252,8 zurückgewonnen. — Bei der gleichen Behandlung einer Lösung von 1,3 g der Thetinsäure in 5,5 ml 1-n. Natronlauge und 25 ml Wasser trat keine sichtbare Veränderung ein, und nach dem Erkalten lieferte die Lösung beim Ansäuern 1,1 g Ausgangsmaterial vom Schmp. 119°–120° wie oben und Äquiv.-Gew. 251,6. — Als dagegen eine Lösung von 1,3 g der Thetinsäure in 30 ml 0,5-n. Natronlauge wie oben erhitzt wurde, ergab die dann schwach opaleszierende Lösung mit 5 ml 5-n. Salzsäure eine schwache Emulgierung, und bei 5-maligem Ausschütteln mit 50 ml Äther wurden ihr Säuren, entsprechend 3,5 + 2 + 1,5 + 1 + 1 = 9 ml 1-n. Natronlauge entzogen. Beim freiwilligen Verdunsten des Äthers wurden als Rückstand 0,4 g einer strahlig kristallinen Masse erhalten, welche aus Phenylglykol vom Schmp. 65°–67° bestand.

Für $C_6H_5CH(OH)CH_2OH = C_8H_{10}O_2$ (138,16):

ber.: C 69,54, H 7,30 %; gef.: C 69,60, H 7,23 %.

Die erhaltene, neutrale Wasserlösung schied nach dem Ansäuern mit 6 ml 2-n. Salzsäure eine unbedeutende Menge Kristallschuppen aus, welche allein und im Gemisch mit der A-Styrylthioglykolsäure bei 87°–89° schmolzen. Aus dem Filtrat davon wurden durch Ausäthern 0,5 g Thiodiglykolsäure vom Schmp. ca. 124°–128° und Äquiv.-Gew. 77,8 gewonnen. Nach Umkristallisieren durch Fällung mit Benzol aus ihrer Lösung in Essigester schmolz diese Säure bei 129°–131° und ergab bei der Analyse:

Für $(HOCOCH_2)_2S = C_4H_6O_4S$ (150,15):

ber.: S 21,35 %, Äquiv.-Gew. 75,1; gef.: S 21,14 %, Äquiv.-gew. 75,6.

Wie bei der A-Säure oben wurden aus 4 g der B-Styrylthioglykolsäure und 7 g Monobromessigsäure 4,4 g einer Salzsäurefällung erhalten, welche hier aus fast reiner Styrylthetinessigsäure bestand, bei 109°–110° unter heftiger Gasentwicklung schmolz und das Äquiv.-Gew. 247,7, ber. 252,3, zeigte. Beim Umkristallisieren von 2 g des Rohprodukts aus 100 ml Wasser wurden 1,5 g fast nadelförmige Prismen vom Schmp. 100°–101° wie oben erhalten.

Für $C_{12}H_{12}O_4S$ (252,28):

ber.: C 57,13, H 4,79, S 12,71 %, Äquiv.-Gew. 252,3,
gef.: » 57,16, » 4,83, » 12,78 » , » » 250,5.

Beim Erhitzen mit Salzsäure usw. verhielt sich diese Thetinsäure wie die entsprechende A-Säure oben.

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Org.-chem. Abteilung des Nobelinstituts der Kgl. Akademie der Wissenschaften, Stockholm.

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Serum fibrinolysin activation in the experimental serum hypersensitiveness of the rabbit

III. Activation experiments with staphylokinase

By Hans von Euler and Leo Heller

By the experiments as described below we continue these reported previously (1, 2), which aimed to check the hypothesis, put forward in a more or less definite form by a variety of authors (e. g. 3, 4) in an attempt to explain the mechanism of the anaphylactic form of hypersensitivity. According to that hypothesis, some kind of activated proteolysis should be considered as constituting the common biochemical basis of the anaphylactic and anaphylactoid hypersensitive reactions. The degeneration of the collagen fibers in the connective tissue, considered by Klinge and his school (5, 11) to represent the primary lesion in the development of the full blossom Aschoff nodule of rheumatic fever, might likewise be tentatively ascribed to a proteolytic effect of the above kind (6). Increased serum proteolysis has already been found in different anaphylactic resp. anaphylactoid reactions in the guinea-pig (3) and in the dog (4), but the general validity of the above correlation, indispensable for unequivocally securing the existence of a causative relation, has to be proved yet. Eventual positive evidence of increased proteolysis — actual or potential, i. e. occurring only under the stimulus of an appropriate activating agent — in rheumatic fever should considerably strengthen both the above assumption of an intimate relation between proteolysis and hypersensitivity and the theory of the anaphylactoid genesis of rheumatism (7, 8).

The broadly conceived idea of activated proteolysis allows for a process of activation of either blood or tissue proteinases. The former, at present known under the names of fibrinolysin (9), or plasmin seems to be much more accessible to an experimental attack, and we thought it therefore advisable to start our investigations with a study of its behaviour. In the search for an adequate experimental model of the rheumatic 'allergy', we choose the relatively well reproducible state of the serum hypersensitiveness of the rabbit (16, 8), the pathogenetic mechanism and the pathological picture of which show many common features with those of the human rheumatic fever. (For a critical review of the latter point compare, however, Waksman (11).)

The rabbit plasma being normally apparently devoid (12, 13) of, or, at its best, showing only an extremely slight degree of proteolytic activity (14), it seemed to us that a successful demonstration of the simultaneous appearance of such an activity and of a state of serum hypersensitiveness, following the intravenous injection of a massive dose of foreign serum, would yield a rather strong argument speaking in favour of the importance of proteolysis in the etiology of that rheumatism-like condition.

As already reported by us (1, 2) no fibrinolysis of an order of magnitude comparable to that regularly shown by the human serum enzyme (10) could so far be obtained with euglobulin preparations derived from sera of normal and horse serum hypersensitive rabbits, showing both humoral (presence of precipitins) and tissue sensitization (positive skin reactions), as well as pathological lesions in the heart of the type described by KLINGE (15) resp. by RICH and GREGERY (16). In the course of these experiments we were able to confirm the fact of a total lack of activity of streptococcal kinase against the supposed rabbit plasma enzyme, for neither of our euglobulin preparations (both from normal and from sensitized rabbit serum) could be definitely activated by a streptokinase preparation, highly active against human serum euglobulin. The narrow species specificity spectrum of streptokinase, acting, as it seems (12, 14) only on human prefibrinolysin, made it necessary to test other, less specific, activating agents. A few experiments (1) making use of prolonged spontaneous activation and of activation by chloroform, respectively, after preliminary removal of the inhibitor-containing albumin fraction of the serum, seemed indicative of the presence of small amounts of activable protease in certain rabbit serum samples. Having taken part of the recent results of GERHEIM and FERGUSON (14) who recently reported a successful activation of proteinases in rabbit and certain other animal sera by staphylococcal filtrates, we tried the latter kind of activating agent (staphylokinase) (17), apparently endowed with a much broader species specificity, than streptokinase, against euglobulin preparations obtained from normal and serum hypersensitized rabbit sera (2).

Methods

Two rabbits, weighing 3 500 and 3 800 gms, respectively, were bled by heart puncture, (sample 60). On the following day either animal was injected intravenously with 35 resp. 38 ml of inactivated sterile horse serum (10 ml per kg body weight (16). The rabbits were bled in weekly intervals, and the sera kept frozen at c:a −10° C. After the bleeding (4 weeks after the horse serum injection) the degree of hypersensitivity and humoral immunity developed was tested by means of:

1. Skin reactivity test, performed by intracutaneous injections of serial tenfold dilutions of horse serum in normal saline (0.1 ml of every dilution resp. of saline control) in the shaved skin of the back of the animal. The reactions were read after 24 hours.
2. Intraarticular injection of 1 ml undiluted horse serum resp. saline (control) into the joint cavity of the right and left knee joint, respectively, of the animals. Eventually occurring swelling of the injected joint was recognized by palpation.
3. Precipitin titration of serum samples by constant antigen ring method (compare e. g. (18), using undiluted antigen and half-capillary microtubes. The test was read twice, after 20 min. and 2 hours respectively. Proteolysis was

measured, as previously done (2), by the fibrinogenolytic technique primarily described by FERGUSON (19) and found to be fairly sensitive (4, 2). The slowness of staphylokinase activation (16) made it, however, necessary to use much longer incubation times than in streptokinase tests. To eliminate bacterial growth, easily occurriog under such conditions, sodium merthiolate (LILY) was added to all solutions used to the final concentration of 0.01 %, this antiseptic having no inhibiting effect on fibrinolysin activation.

The staphylokinase-producing strain of *staphulococcus aureus* used in the tests with rabbit serum enzyme has been chosen on the basis of a screening experiment, in which ten different coagulase-positive and toxin producing strains of that microorganism were tested on their kinase-producing ability.[1] Strain N:o 1217/50, which has shown the greatest kinase activity when tested against normal human serum euglobulin in a fibrinogenolytic test, was used throughout the experiments with rabbit serum material.

The modified fibrinogenolytic technique, finally adopted by us in the staphylokinase work, consisted in mixing 0.2 ml aliquots of serial twofold dilutions of the rabbit serum euglobulin preparations to be tested (prepared by ammonium sulphate precipitation to 30 % saturation and resuspended in one fifth the original serum volume of phosphate-buffered saline (pH = 7.6)), with 0.1 ml amounts of a Seitz filtrate of a 48 hours broth culture of *staphylococcus aureus* (strain 1217/50). After 60 minutes' incubation at 37° C. 0.2 ml aliquots of a 0.5 % solution of bovine plasma fibrinogen ("ARMOUR LABORATORIES") were added to the tubes. The racks were shaken and incubated for 48 hours in a water-bath of 37° C. Spontaneous clotting occurred rather quickly in a part of tubes (coagulase action), but in the presence of active proteinase the gel formed dissolved slowly on prolonged incubation. After 48 hours the racks were taken out and one drop of thrombin solution, containing a ca. 10-fold multiple of the minimal quantity necessary to clot the amount of fibrinogen initially present was added to each tube. The presence resp. absence of gelation was recorded after 20 minutes standing at room temperature. Both the fibrinogen and thrombin solutions were prepared in the same phosphate-saline buffer, containing merthiolate. Control series, in which saline was substituted for the staphylococcal filtrate, were included in the experiments.

Results

Only one of two rabbits injected with horse serum proved to be distinctly hypersensitized against his antigen, showing:

1. a positive skin test down to the dilution 1 : 50 of the horse serum.
2. a distinct swelling of the knee joint injected with the horse serum (no swelling in the control joint, injected with the same volume of saline).
3. a serum precipiting titer of 1 : 16 against undiluted horse serum used as antigen. That titer has heen reached already in the 1st serum sample, taken 1 week after the infection of antigen, and remained fairly constant throughout the duration of the experiment (4 weeks period).

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[1] The authors are greatly indebted to Dr. K. ALIN from the State Bacteriological Laboratory, Stockholm, for kindly supplying us with the strains in question.

Table I.

Serum			Pre-cipitin Titer	Fibrinolysin Titration				
				with staphylococcal extract			without staphylocc. extract	
Rabbit No.	Sample No.	No. of Days after serum injection		Tube 1 (undil. eugl.)	Tube 2 (eugl. dil. 1 : 2)	Tube 3–6 (dil. 1 : 4–1 : 32)	Tube 1 (undil. eugl.)	Tubes 2–6 (dil. 1 : 2–1 : 32)
Hypersensitized rabbit:								
1	0 (norm.)	0 (before)	0	dissolved	clot	clot	part. dissolv.	clot
	1	7	1 : 16	»	»	»	»	»
	2	14	»	part. dissolv.	»	»	»	»
	3	22	»	dissolved	part. dissolv.	»	»	»
	4	28	»	»	clot	»	»	»
	5	29	1 : 8	dissolved	dissolved	»	»	»
Normal rabbits:								
2				dissolved	clot	clot	part. dissolv.	clot
3				»	dissolved	»	»	»
4				»	clot	»	»	»
5				part. dissolv.	part. dissolv.	»	clot	»
6				clot	clot	»	»	»
7				dissolved	part. dissolv.	»	part. dissolv.	»

The other rabbit gave only a slightly positive skin reaction with undiluted antigen, no distinct swelling of the joint injected with the latter, and no distinct precipiting reaction. The serum sample coming from this animal were therefore discarded and the fibrinolysin activation test performed only with euglobulin preparations derived from serum samples of the first rabbit and of normal, untreated animals.

Altogether 7 normal rabbit sera and 5 serum samples from the hypersensitized animal, taken 7, 14, 22, 28 and 29 days, respectively, after the intravenous injection of horse serum, were tested by the modified fibrinogenolytic method described above, both in the presence and in the absence of staphylococcal filtrate. The last (29 days') sample was taken 24 hours after the intracutaneous and intraarticular challenge with the sensitizing antigen.

The results, summarized in the table, show rather clearly, that staphylococcal extracts have a slight activating effect on the protease present in a certain proportion of rabbit sera. The amounts of the latter must, however, be relatively low, for a two- or in some cases four-fold dilution of the 5-fold concentrated rabbit euglobulin was generally sufficient for diluting out the fibrinogenolytic action of the latter, while analogous preparations of human euglobulin were active down to the dilution of 1 : 64, as found in screening experiments with staphylococcal extracts from different strains. That confirms the results obtained by GERHEIM and FERGUSON (14). Prolonged incubation of rabbit serum euglobulin with fibrinogen without addition of any activator also seems to bring forth a certain degree of activation of the protease, potentially present in the rabbit serum. This activation is, however, still weaker than that produced by staphylokinase (see table). No definite difference between the behaviour of normal and hypersensitized rabbit serum in respect to their potential proteolytic capacity could be demonstrated by the technique used. It is, however, possible, that here may exist activating agents more appropriate for unveiling the inherent proteolytic capacity of rabbit sera. It would be interesting to test the last of the so far known fibriniolysin activating agents, the 'fibrinokinase' of PERMIN and ASTRUP (20) present in tissue cells. So far, no unequivocal positive evidence, supporting the theories of a proteolytic pathogenesis of anaphylaxis or of rheumatic fever can be derived from the results presented.

Grateful acknowledgement is made to Ing. ROGNE ANDER for valuable technical assistance.

SUMMARY

Only slight fibrinogenolytic activity was obtained in activation experiments with rabbit serum euglobulin and staphylokinase containing staphylococcal extracts. No difference was found between the behaviour of normal (untreated) and serum hypersensitive rabbit plasma euglobulin in the above respect.

The importance of the failure to demonstrate an activation of serum proteolysis in the experimental serum hypersensitiveness of the rabbit for the validity of the proteolysis activation theories of the pathogenesis chronic of anaphylaxis and rheumatism is discussed.

Stockholm, University, Vitamin Institute.

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Use of different alkali carbonates in the methylation of acetylacetone

By ARNE BRÄNDSTRÖM

With 8 figures in the text

As has been preliminarily reported (1) an investigation of the kinetics of the methylation of acetylacetone with the aid of various alkali carbonates and methyl iodide has been carried out.

The mathematical treatment of the experimental material has indicated that the monomethylation reaction of acetylacetone is of the first order, with the reaction velocity directly proportional to the concentration of methyl iodide in the case of all alkali carbonates.

The methylation reaction of methylacetylacetone seems to be of the first order, with the reaction velocity directly proportional to the concentration of methyl iodide for potassium carbonate, but of the second order with the reaction velocity proportional to both the concentration of methyl iodide and methylacetylacetone for rubidium and cesium carbonates.

For the mathematical deductions the following symbols are introduced:

Reaction $A \xrightarrow{E} B \xrightarrow{E} C$;

a, b, c, and e are the concentrations of A, B, C, and E;
a_0 and e_0 are the initial concentrations of A and E;
t is the time in minutes;
k_1 and k_2 are the reaction velocity constants.

When both reactions are of the first order with the reaction velocity directly proportional to e, the following differential equations are valid:

$$\frac{da}{dt} = -k_1 \cdot e; \qquad \frac{dc}{dt} = k_2 \cdot e \tag{1}$$

Taking into consideration that $a + b + c = a_0$, and $e + b + 2c = e_0$, we obtain

$$\frac{db}{dt} = -\frac{da}{dt} - \frac{dc}{dt} = e(k_1 - k_2); \tag{2}$$

$$\frac{de}{dt} = -\frac{db}{dt} - \frac{2\,dc}{dt} = -e(k_1 + k_2); \tag{3}$$

Integration of eq. (3) gives

$$\ln \frac{e}{e_0} = - t(k_1 + k_2). \tag{4}$$

From this it is evident that the reaction proceeds with the same velocity as a simple first order reaction having its velocity constant equal to $(k_1 + k_2)$.

As k_2 can be readily obtained from the reaction $\mathrm{B} \xrightarrow{\mathrm{E}} \mathrm{C}$ both k_1 and k_2 can be evolved.

For synthetical purposes the ratio of the concentration of C to that of B is of great interest. This can be evolved in the following way:

From eq. (1) and (2) we obtain

$$\frac{dc}{db} = \frac{k_2}{k_1 - k_2}, \quad \text{or} \quad \frac{c}{b} = \frac{k_2}{k_1 - k_2}. \tag{5}$$

From the moment that all A has disappeared it is obvious that the reaction proceeds with its velocity constant equal to k_2.

When the first reaction is of the first order, with its velocity directly proportional to e, and the second of the second order, with its velocity proportional to both b and e, the following equations are valid:

$$\frac{da}{dt} = - k_1 \cdot e; \qquad \frac{dc}{dt} = k_2 \cdot b \cdot e \tag{6}$$

Taking into consideration that $a + b + c = a_0$ and $e + b + 2c = e_0$, we obtain

$$\frac{db}{dt} = - \frac{da}{dt} - \frac{dc}{dt} = e(k_1 - k_2 \cdot b) \tag{7}$$

$$\frac{de}{dt} = - \frac{db}{dt} - 2\frac{dc}{dt} = - e(k_1 + k_2 \cdot b) \tag{8}$$

From eq. (7) we get on integration,

$$\ln \frac{k_1 - k_2 b}{k_1} = - k_2 \int_0^t e \cdot dt \tag{9}$$

By plotting e against t, we can obtain $\int_0^t e \cdot dt$ by a graphical integration, k_2 being readily obtainable from the reaction $\mathrm{B} \xrightarrow{\mathrm{E}} \mathrm{C}$, and from this we can obtain $\frac{k_2}{k_1} b$ at various times.

From eq. (8) we obtain

$$\frac{de}{dt} = - k_1 \cdot e\left(1 + \frac{k_2}{k_1} b\right),$$

which on integration gives

$$\ln \frac{e}{e_0} = -k_1\, t - k_1 \int_0^t \frac{k_2}{k_1} b \cdot dt \qquad (10)$$

By plotting $\frac{k_2}{k_1} b$ against t, $\int_0^t \frac{k_2}{k_1} b \cdot dt$ can be obtained by a graphical integration and from this we obtain k_1.

Experimental

Procedure and apparatus

The progress of methylation was followed by the determination of the iodide-ion concentration.

The determinations were carried out in two different scales, a macro scale and a micro scale, with amounts of diketone of 10 millimols and 0.1 millimol resp. The micro method is the one preferred, but in lengthy experiments better results are obtained by the macro method, due to the fact that more efficient condensers can be employed by this method.

The determinations were carried out at the reflux temperature of the mixture. The rate of refluxing was kept constant by immersing the flasks into a thermostat kept at 65°. Bumping was avoided by filling the flasks with fine Pyrex wool.

The macro scale

Pyrex wool and a carbonate were placed in 50-ml flasks. The flasks were fitted with efficient reflux condensers, and cooled in an ice bath, the reaction mixture being pipetted out to each flask. The mixture was then refluxed by immersing the flasks into the thermostat kept at 65°, alcohol at −15° being circulated through the condensers. At fixed intervals a flask was taken, and the contents were rapidly washed into a 250-ml volumetric flask with 2 % acetic acid and made up to the mark. 50, or 100 ml of the solution was pipetted out, 4 ml of glacial acetic acid added, and the solution titrated with 0.1 normal silver nitrate, 10 drops of a 0.1 % eosine solution being used as an indicator.

The micro scale

This method has previously been described (1). The only difficulty in this method consists in breaking the flasks without splashing but this can easily be done by placing the flasks into a short-stemmed funnel, a sharp rod of stainless steel being introduced into the neck, and the bottom of the flask broken by being forced through. The contents can then easily be washed into the Erlenmeyer flask.

Each method usually gives good results, but about a fourth of the single determinations must be rejected as being very erroneous (over 20 %). This is probably due to the fact that bumping cannot be avoided in every case, but when this occurs, the values are invariably very erroneous.

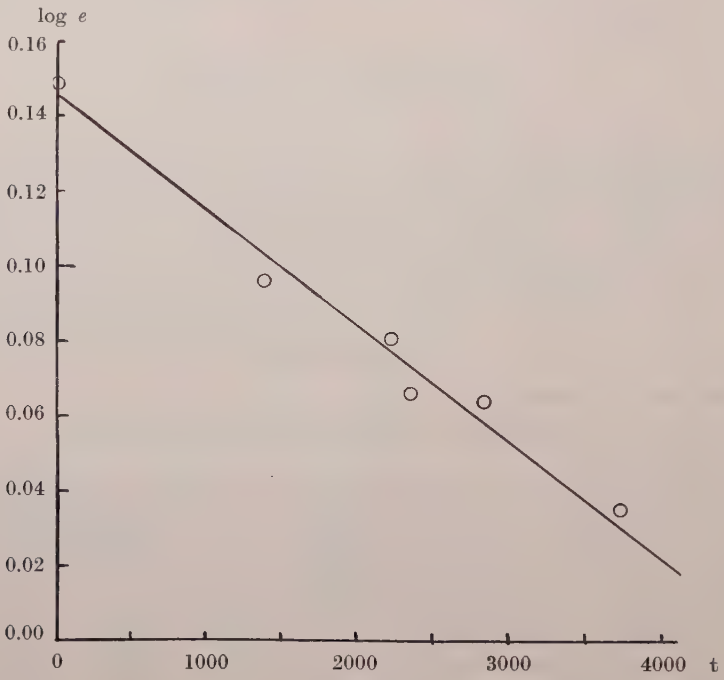

Fig. 1.

Table 1

The monomethylation reaction with sodium carbonate

t	m. ekv. I^-	e	log e
0	0	1.409	0.1489
1380	0.0324	1.247	0.0959
2220	0.0414	1.202	0.0803
2340	0.0490	1.164	0.0660
2820	0.0502	1.158	0.0637
3770	0.0650	1.084	0.0350
$k_1 + k_2 = 7 \cdot 10^{-5}$ at 761—763 mm Hg			

Of course, there are many systematic errors in the determinations, but the results obtained by the kinetic study of the alkylation are of very great value for the preparative use of the reaction, which may be illustrated by the syntheses of methylacetylacetone and dimethylacetylacetone.

The carbonates used were the commercial granular products. The acetylacetone and the methyl iodide were redistilled pure products. The methylacetylacetone in the macro scale was a crude product, but in the micro scale a product that had been purified over the copper derivate by repeated recrystallisation. The acetone was a pure commercial product that has been dried with anhydrous potassium carbonate.

Sodium carbonate

The micro method was used. As this reaction is very slow, it was not studied closely, and only the monomethylation reaction was examined. 0.200 ml of an acetone solution containing 40.0 mg of methyl iodide and 10.0 mg of acetylacetone was used in each flask together with 23 mg of anhydrous sodium carbonate. The reaction time was extended to 62 hours, but a slight discolouration occurred. The results are shown in Table 1 and Fig. 1.

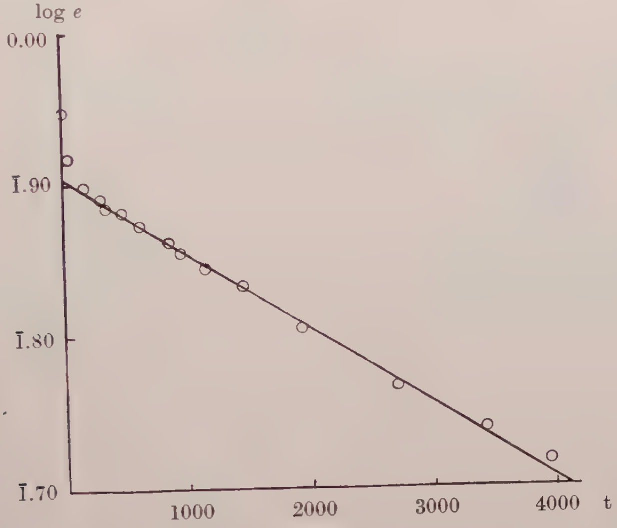

Fig. 2.

Table 2

The dimethylation reaction with potassium carbonate

t	m. ekv. I^-	*e*	log *e*
0	0	0.881	$\bar{1}.9448$
60	1.08	0.827	$\bar{1}.9175$
180	1.78	0.792	$\bar{1}.8987$
300	2.06	0.778	$\bar{1}.8910$
360	2.28	0.767	$\bar{1}.8848$
480	2.38	0.762	$\bar{1}.8820$
630	2.68	0.747	$\bar{1}.8733$
840	3.06	0.728	$\bar{1}.8621$
960	3.28	0.717	$\bar{1}.8555$
1140	3.64	0.699	$\bar{1}.8445$
1460	4.00	0.681	$\bar{1}.8331$
1930	4.86	0.638	$\bar{1}.8048$
2760	5.93	0.584	$\bar{1}.7664$
3430	6.68	0.547	$\bar{1}.7380$
3960	7.15	0.523	$\bar{1}.7185$
$k_2 = 1.15 \cdot 10^{-4}$ at 740—745 mm Hg			

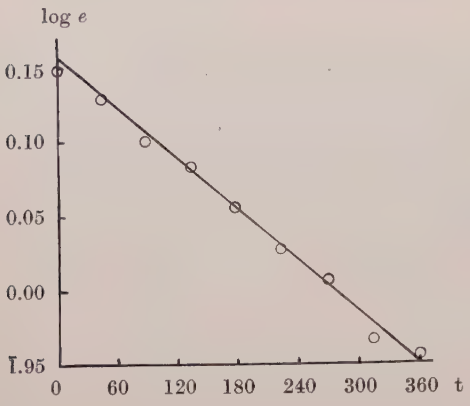

Fig. 3.

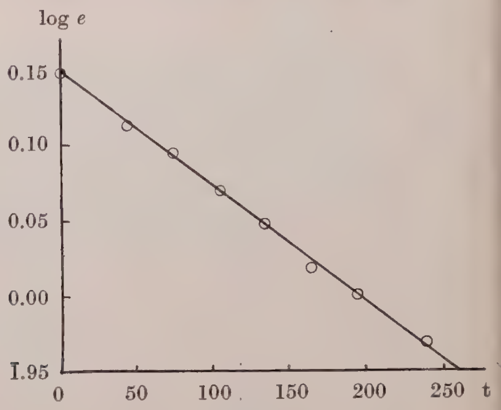

Fig. 4.

Table 3

The monomethylation reaction with potassium carbonate

The macro method

t	m. ekv. I^-	e	log e
0	0	1.409	0.1489
45	1.30	1.344	0.1284
90	3.05	1.257	0.0993
135	4.01	1.209	0.0824
180	5.49	1.135	0.0550
225	6.92	1.063	0.0265
270	7.95	1.012	0.0052
315	9.70	0.924	$\bar{1}.9657$
360	10.15	0.902	$\bar{1}.9552$

$k_1 + k_2 = 1.29 \cdot 10^{-3}$ at 730—733 mm Hg

Table 4

The monomethylation reaction with potassium carbonate

The micro method

t	m. ekv. I^-	e	log e
0	0	1.409	0.1489
45	0.0220	1.299	0.1136
75	0.0336	1.241	0.0938
105	0.0474	1.172	0.0689
135	0.0586	1.116	0.0477
165	0.0737	1.041	0.0175
195	0.0822	0.998	$\bar{1}.9991$
240	0.0959	0.930	$\bar{1}.9685$

$k_1 + k_2 = 1.77 \cdot 10^{-3}$ at 775—777 mm Hg

Potassium carbonate

The macro method was used for both the mono- and the dimethylation reaction. 20.0 ml of an acetone solution containing 1.00 g of acetylacetone and 4.00 g of methyl iodide, or 1.14 g of methylacetylacetone and 2.50 g of methyl iodide was used together with 3.00 g of anhydrous potassium carbonate. From the experiments it is obvious that the methylacetylacetone contained a considerable amount of acetylacetone, but as the reaction is of the first order, this is not very disadvantageous as the reaction velocity constant can easily be calculated.

A parallel determination was carried out with the micro method on the monomethylation reaction. In this case 0.200 ml of an acetone solution containing 10.0 mg of acetylacetone and 40.0 mg of methyl iodide was used in each flask together with 30 mg of anhydrous potassium carbonate. As the atmospheric pressure in the two runs was very different, the influence of this pressure on the reflux temperature and consequently on the velocity constant can be calculated and found to be of the order of 1 % for a change of 1.5 mm Hg for small variations in the atmospheric pressure. This value is taken whenever the reaction velocity constants are measured at different pressures.

The results are shown in Fig. and Tables 2–4.

Rubidium carbonate

The micro method was used for both the mono- and dimethylation reaction. 1.000 ml of an acetone solution containing 10.0 mg of acetylacetone and 40.0 mg of methyl iodide, or 11.4 mg of methylacetylacetone and 40.0 mg of methyl iodide was used together with 50 mg of anhydrous rubidium carbonate. With a more concentrated solution such as that used with potassium carbonate reproducible results could not be obtained. The latter are shown in Fig. and Tables 5–6.

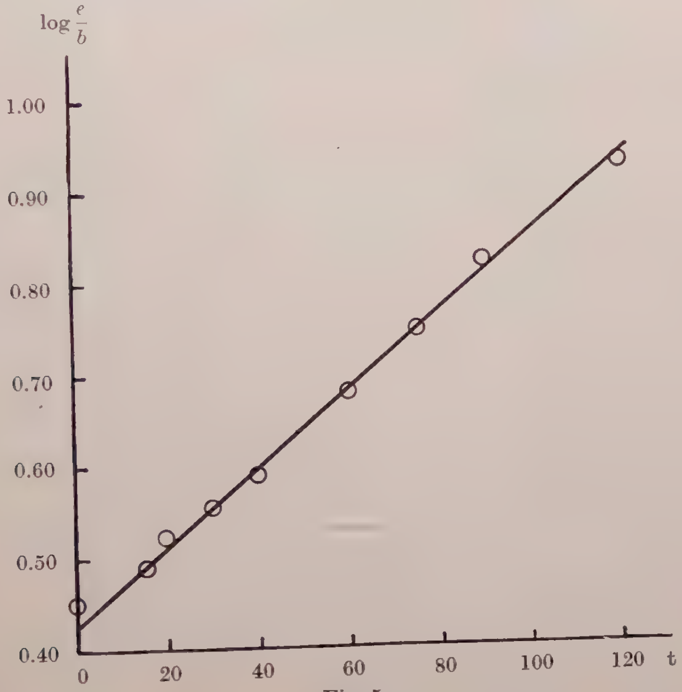

Fig. 5.

Table 5

The dimethylation reaction with rubidium carbonate

t	m. ekv. I^-	e	b	log e/b
0	0	0.2818	0.1000	0.4499
15	0.0130	0.2688	0.0870	0.4900
20	0.0218	0.2600	0.0782	0.5218
30	0.0294	0.2524	0.0706	0.5533
40	0.0370	0.2448	0.0630	0.5895
60	0.0520	0.2298	0.0480	0.6801
75	0.0606	0.2212	0.0394	0.7493
90	0.0660	0.2158	0.0340	0.8026
120	0.0760	0.2058	0.0240	0.9333
$k_2 = 5.34 \cdot 10^{-2}$ at 768 mm Hg				

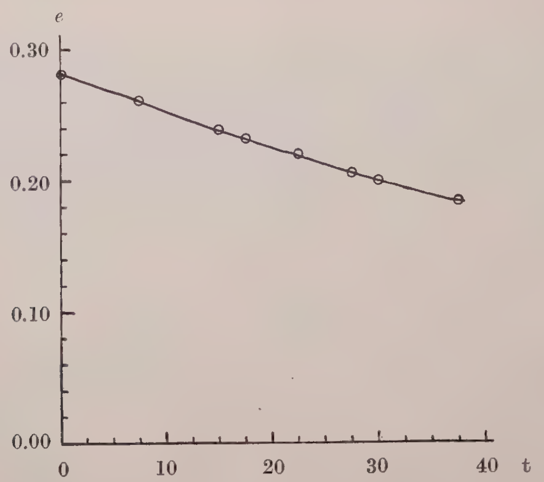

Fig. 6 a.

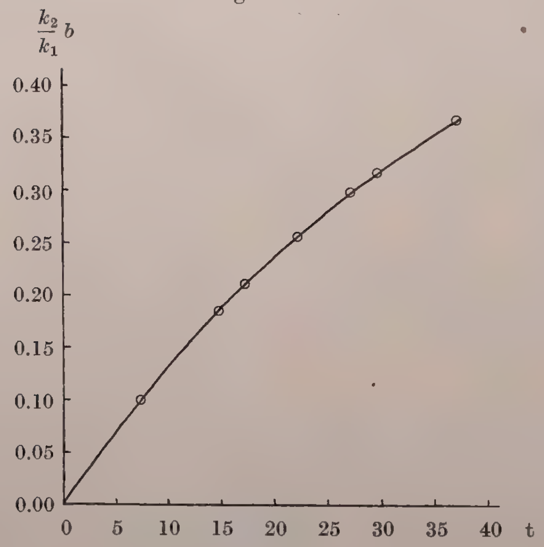

Fig. 6 b.

Table 6

The monomethylation reaction with rubidium carbonate

t	m. ekv. I^-	e	$\int_0^t e \cdot dt$	$k_2/k_1 \cdot b$	$t + \int_0^t k_2/k_1 \cdot b \cdot dt$	$k_1 \cdot 100$
0	0	0.2818	0	0	0	
7.5	0.0208	0.2610	2.04	0.100	7.88	0.97
15	0.0436	0.2382	3.91	0.184	16.44	1.02
17.5	0.0506	0.2312	4.50	0.209	19.43	1.02
22.5	0.0624	0.2194	5.63	0.254	25.59	0.98
27.5	0.0764	0.2054	6.69	0.294	31.96	0.99
30	0.0828	0.1990	7.20	0.313	35.22	0.99
37.5	0.0984	0.1834	8.63	0.362	45.26	0.95
$k_1 = 0.99 \cdot 10^{-2}$ at 764 mm Hg						

Cesium carbonate

The micro method was used for both the mono- and the dimethylation reaction. 0.200 ml of an acetone solution containing 10.0 mg of acetylacetone and 40.0 mg of methyl iodide, or 1.000 ml of an acetone solution containing 11.4 mg of methylacetylacetone and 40.0 mg of methyl iodide together with 70 mg of anhydrous cesium carbonate. *With a more concentrated solution of methylacetylacetone such as that used with potassium carbonate the reaction was too violent and could not be kept under control.*

The results are shown in Fig. and Tables 7–8.

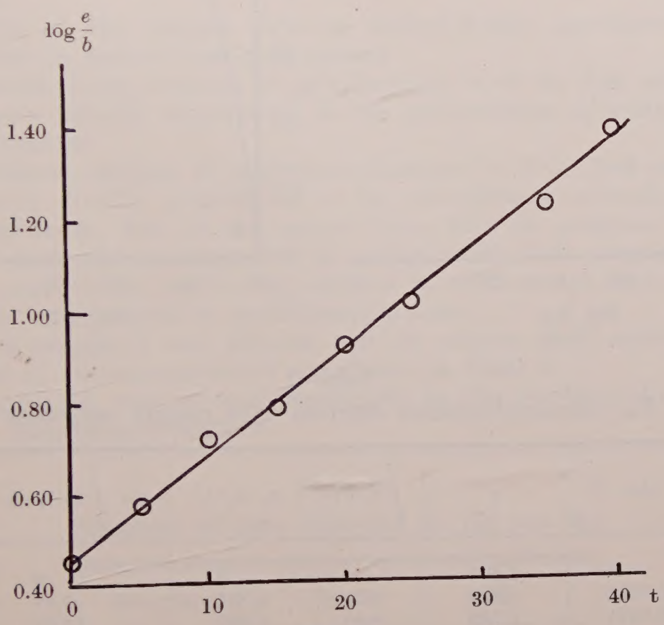

Fig. 7.

Table 7

The dimethylation reaction with cesium carbonate

t	m. ekv. I^-	e	e	log e/b
0	0	0.2818	0.1000	0.4499
5	0.0338	0.2480	0.0662	0.5736
10	0.0570	0.2248	0.0430	0.7183
15	0.0640	0.2178	0.0360	0.7818
20	0.0750	0.2068	0.0250	0.9177
25	0.0804	0.2014	0.0196	1.0117
35	0.0884	0.1934	0.0116	1.2220
40	0.0924	0.1894	0.0076	1.3966
$k_2 = 2.85 \cdot 10^{-1}$ at 765 mm Hg				

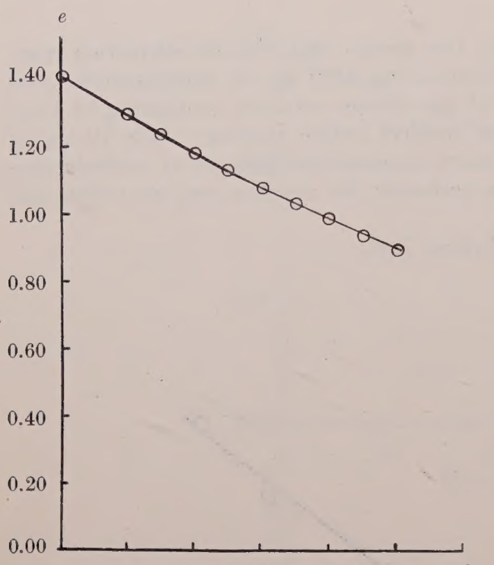

Fig. 8 a.

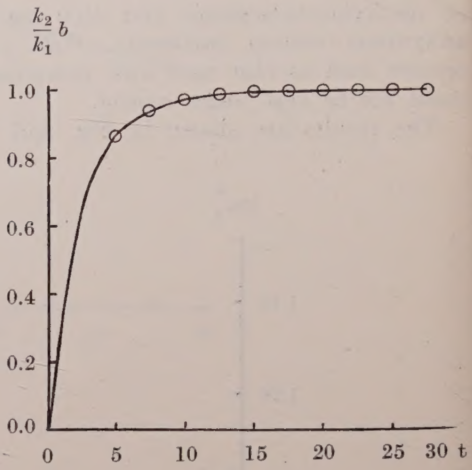

Fig. 8 b.

Table 8

The monomethylation reaction with cesium carbonate

t	m. ekv. I^-	e	$\int_0^t e \cdot dt$	$k_2/k_1 \cdot b$	$t + \int_0^t k_2/k_1 \cdot b \cdot dt$	k_1 100
0	0	1.409	0	0	0	
5	0.0232	1.293	6.76	0.865	7.87	1.09
7.5	0.0341	1.239	9.92	0.947	12.64	1.01
10	0.0470	1.174	12.94	0.978	17.55	1.04
12.5	0.0560	1.129	15.82	0.991	22.51	0.98
15	0.0678	1.070	18.57	0.996	27.49	0.97
17.5	0.0763	1.028	21.19	0.998	32.48	0.97
20	0.0856	0.981	23.70	0.999	37.48	0.97
22.5	0.0958	0.930	26.09	0.999	42.48	0.97
25	0.1054	0.882	28.35	1.000	47.48	0.98

$k_1 = 1.00 \cdot 10^{-2}$ at 771 mm Hg

The preparation of methylacetylacetone and dimethylacetylacetone

Methylacetylacetone: A mixture of 1 mole of acetylacetone, 1.2 moles of methyl iodide, 2 moles of potassium carbonate, and 500 ml of acetone was refluxed with stirring for 30 hours. The acetone was distilled off, the residue acidified with diluted sulphuric acid and extracted repeatedly with ether. The ethereal extracts were dried with anhydrous sodium sulphate, and then distilled. The fraction boiling at 165–170° was collected as methylacetylacetone containing about 10 %

of dimethylacetylacetone. Yield: over 90 % of this crude product. This can be purified over the copper salt in the usual way.

Dimethylacetylacetone: A mixture of 1 mole of acetylacetone, 3 moles of methyl iodide, 4 moles of anhydrous potassium carbonate, and 1 l of acetone was refluxed with stirring for 150 hours. The reaction product was worked up as above, giving an excellent yield of dimethylacetylacetone boiling at 74–75°/15 mm, which solidified on cooling to a little below room temperature.

SUMMARY

The kinetics of the reaction between methyl iodide, acetylacetone, and an alkali carbonate in acetone has been studied.

The monomethylation reaction of acetylacetone is of the first order with the reaction velocity directly proportional to the concentration of methyl iodide for all alkali carbonates.

The methylation reaction of methylacetylacetone is of the first order with the reaction velocity directly proportional to the concentration of methyl iodide for potassium carbonate, but of the second order with the reaction velocity proportional to both the concentration of methyl iodide and methylacetylacetone for rubidium and cesium carbonates, at least in solutions not more concentrated than 0.1 mole with respect to methylacetylacetone.

The reaction velocity is very different with the various alkali carbonates.

The results of the measurements are shown in Table 9.

The utility of the reaction is demonstrated by the synthesis of methylacetylacetone and dimethylacetylacetone.

Table 9

Summary of data corrected to 760 mm Hg

Carbonate	k_1	k_2
Na	$7 \cdot 10^{-5}$	—
K	$1.47 \cdot 10^{-3}$	$1.25 \cdot 10^{-4}$
Rb	$0.96 \cdot 10^{-2}$	$5.05 \cdot 10^{-2}$
Cs	$0.92 \cdot 10^{-2}$	$2.76 \cdot 10^{-1}$

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Laboratory of Organic Chemistry, University of Uppsala.

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